REMARKS

In response to the Office Action of June 6, 2004, Applicants have amended the claims, which when considered with the following remarks, is deemed to place the present application in condition for allowance. Favorable consideration of all pending claims is respectfully requested.

Claims 1 and claims dependent thereon, have been rejected under 35 U.S.C.§ 112, first paragraph, as allegedly violative of the written description requirement. According to the Examiner, the recitation of "a protein comprising amino acids 96-118 of SEQ ID NO:8, allowing up to four mismatches" allegedly does not find support in the specification as originally filed and therefore allegedly constitutes new matter. Office Action, page 3.

To comply with the written description requirement, it is not necessary that the application describe the invention *ipsis verbis*. *In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971). What *is* required is that an ordinarily skilled artisan recognize from the disclosure that applicants invented the subject matter of the claims, including the limitations recited therein. *Smith*, 481 F.2d at 915, 178 USPQ at 284. Thus, it has been well settled that the written description requirement of 35 U.S.C. § 112, first paragraph, can be satisfied without express or explicit disclosure of a later-claimed invention. *See e.g. In re Herschler*, 591 F.2d 693, 700, 200 USPQ 711, 717 (CCPA). Applicants do not agree therefore, that the limitation, "a protein comprising amino acids 96-118 of SEQ ID NO:8, allowing for up to four mismatches" does not find support in the application as filed.

Nonetheless, in order to advance prosecution of this application, claim 1 has been amended so that it no longer recites "a protein comprising amino acids 96-118 of SEQ ID NO:8, allowing for up to four mismatches." In its place, the language "a protein having greater than 80% sequence identity to amino acids 96 to 118 of SEQ ID NO:8" has been substituted.

Applicants reserve the right to file one or more continuation applications directed to the subject matter deleted from claim 1. Withdrawal of the rejection of claim 1 and claims dependent thereon, under 35 U.S.C.§ 112, first paragraph, as allegedly reciting new matter, is therefore warranted.

Claims 1, 5-10, 13-23 and 50 remain rejected under 35 U.S.C.§ 112, first paragraph, as allegedly violative of the written description requirement. On page 4 of the Office Action, the Examiner sets forth a basis for the rejection as "first, while the language refers to a conserved structural feature of the disclosed sequence, namely amino acids 96-118 of SEQ ID NO:8, that are correlated with conserved structural features of the HAL3 sequences of the prior art, neither the specification nor the prior art indicates whether the conserved structural feature is correlated with any specific function, or with a function required to practice Applicants' claimed invention. The Examiner has asserted that Ferrando et al. (1995) "Regulation of Cation Transport in *Saccharomyces cerevisiae* by the Salt Tolerance Gene *HAL3*" *Mol. Cell. Biol. 15*(10):5470-5481, indicates that amino acids outside of the corresponding region in HAL3 are required for HAL3 salt tolerance (page 5474 column 1, second full paragraph and p5473 figure 1). However, it is respectfully submitted that an article published after Ferrando et al., i.e., Rodriguez et al. (1996) "CtCdc55p and CtHal3p: Two Putative Regulatory Proteins from *Candida tropicalis* with long Acidic Domains" *Yeast 12:*1321-1329 (provided herewith as Exhibit 1), contradicts the conclusion by Ferrando et al. and thus the position of the Examiner.

Rodriguez et al. teach that the acidic C-terminal domain is found in a wide variety of proteins that are not involved in salt tolerance and that are unrelated to the yeast HAL3 protein, such as nuclear proteins (e.g. the centromere protein CENP-B, non-histone proteins HMG-1,2, nucleolin and nucloplasmin or the UBF transcription factor) or microsomal proteins

(calsequestrin or calreticulin) (*see* page 1326, second column, last paragraph to page 1327, second column, line 4). For these proteins, the acidic domain is postulated to function in chromatin unfolding or calcium binding. In particular, Rodriguez et al. suggests that the acidic domain of the *Candida* HAL3 is involved in protein-protein interactions. Rodriguez et al. suggest that the acidic region forms a module for interactions between subunits of protein complexes (page 1327, 2nd column, lines 38-41), in particular for binding to the catalytic subunit of the phosphatase (page 1327, 2nd column, lines 33-35). Rodriguez et al. also teach that the N-terminal half of the proteins ScHAL3, CtHAL3 and YKLW088w are not significantly homologous to each other (page 1323, 2nd column, results & discussion, lines 22-24 and figure 2). Figure 2 of Rodriguez et al. furthermore shows that the homology between the acidic region of CtHAL3 (residues 463 to 531) or ScHAL3 (residues 496 to 562) and YKL088w (residues 508 to 571) is also low (24.4 and 29.9% sequence identity), whereas the homologies for the second half of the proteins but without the acidic region (ScHAL3: residues 260-495; CtHAL3: residues 264-462; YKL088w: residues 305-507) are considerably higher: 44.5 and 37.7% sequence identity (*See* Exhibit 2).

Rodriguez et al. furthermore teach that YKL088w could complement the salt sensitivity of a *hal3::LEU2 S. cerevisiae* strain (page 1326, first column, second paragraph). Since it is well accepted in the art that structural conservation among proteins relates to functional conservation, a person skilled in the art would have reasonably believed at the time the present application was first filed, that the region with the highest sequence similarity would be responsible for the function which is common between these proteins. Thus, a person skilled in the art would have reasonably believed that the C-terminal half of the proteins, without the acidic region, would most likely be responsible for the halotolerance.

It was also based on this assumption that Espinoza-Ruiz et al. (The Plant Journal 20, 529-539; 1999), submitted herewith as Exhibit 3, named the isolated plant genes HAL3a and HAL3b and tested whether the plant HAL3a conferred salt tolerance to yeast, which was indeed the case Specifically, Espinosa-Ruiz et al. disclose: "[o]ne striking difference between yeast HAL3 and A*rabidopsis* AtHAL3 is the presence in the fungal protein of a long acidic tail which has been reported to be essential to improve NaCl tolerance (Ferrando et al. 1995) and to improve the growth of *sit4* mutants (Di Como et al., 1995). Accordingly, we have included in our complementation studies both a truncated yeast HAL3, devoid of the acidic tail, and a chimeric AtHAL3 where the yeast acidic tail was fused to the AtHAL3 coding sequence. Figure 5 shows the lithium tolerance of the yeast strain RS48 (hal3 null mutant) transformed with different constructions including: yeast HAL3, yeast HAL3 without the acidic tail, AtHAL3a, and a chimeric gene consisting of AtHAL3a fused with the yeast HAL3 acidic tail...Complementation of lithium tolerance with a plasmid containing a yeast *HAL3* showed little dependence on the presence of an acidic tail." Espinosa-Ruiz, page 532, column 2.

Thus, the rejection of claims predicated on the region conferring salt tolerance residing in the C-terminal domain is inaccurate based on the teachings by Rodriguez et al. and Espinosa-Ruiz et al. and should be reconsidered by the Examiner.

On page 5 of the Office Action, the Examiner alleges that the current claim 1 is not limited to a conserved structural feature of the disclosed sequence, namely amino acids 96-118 of SEQ ID NO: 8, but instead allows for up to four unspecified mismatches. As currently amended, claim 1, part (c) recites in relevant part: "a DNA molecule comprising a nucleotide sequence hybridizing with the complementary strand of a nucleotide sequence as defined in (a) or (b) under stringent hybridization conditions of hybridization in 4X SSC at 65° C, followed by

washing in 0.1X SSC at 65° C, or hybridization in 50% formamide, 4X SSC at 42° C, followed by washing in 0.1X SSC; wherein the nucleotide sequence encodes a protein having greater than 80% sequence identity to amino acids 96 to 118 of SEQ ID NO:8." Part (d) of claim 1 has been deleted. Applicants reserve the right to file one or more continuation applications directed to the subject matter deleted from claim 1. Support for the language of part (c) of claim 1 may be found throughout the specification, e.g., page 18, line 31, to page 19, line 1.

In view of the amendments to claim 1 and the foregoing remarks, withdrawal of the rejection of claims 1, 5-10, 13-23 and 50 under the written description requirement of 35 U.S.C. 112, first paragraph is respectfully requested.

Claims 1, 5-10, 13-23 and 50 have been rejected as allegedly failing to comply with the enablement requirement of 35 U.S.C.§ 112, first paragraph. According to the Examiner, the specification does not disclose a specific function for the polypeptide encoded by SEQ ID NO: 7, and the specification does not disclose the effect of transforming a plant or cell with the claimed DNA sequence. Applicants respectfully traverse the rejection for the following reasons: Example 4 of the specification clearly states on pages 72 and 73 that the HAL3 gene is useful for conferring salt tolerance on plants and to improve plant growth under conditions of salt stress. In the description on p34, lines 5-8, it is stated that "...overproduction of the cell cycle interacting protein of the invention enhances growth and results in cell division to be less sensitive to an arrest caused by environmental stress such as salt, ...". Similar statements are made on page 42, lines 2-4, page 49, lines 8-11, page 61, lines 6-19, page 62, lines 5-8 and line 29 to page 63, line 5. Ferrando et al. demonstrated that the related yeast gene is involved in yeast salt tolerance and later studies by Espinoza-Ruiz et al. (Exhibit 3) and by Yonamine et al. (2004) "Overexpression of *NtHAL3* genes confers increased levels of proline biosynthesis and the enhancement of salt

tolerance in cultured tobacco cells" *J. Exp. Bot.* *55*(396): 387-395, provided herewith as Exhibit 4, showed that the plant homologues of yeast HAL3 perform the same function. Espinoza-Ruiz et al. specifically teach "[t]ransgenic *Arabidopsis* plants, with gain of *AtHAL3a* function, show altered growth rates and improved tolerance to salt and osmotic stress" (abstract, last sentence), and Yonamine et al., et al. specifically teach "Overexpression of NtHAL3a improved salt, osmotic, and lithium tolerance in cultured tobacco cells" (abstract lines 20-22). The fact that some of the references submitted as Exhibits 1 through 4 were published after the original filing date of the present application is appropriate since the teachings of the published references do not add to the teachings of the present specification and demonstrate results using techniques available at the relevant time. *See* Gould v. Quigg, 822 F.2d 1074, 3 USPQ 2d 1302 (Fed. Cir. 1987).

The Examiner has posited on page 7 that the record indicates that the region spanning residues 96-118 of SEQ ID NO: 8, corresponding to amino acids 376-398 in HAL3 is highly conserved, and that the HAL3 sequence differs from SEQ ID NO: 8 in 5 amino acids in this region. In this respect, the Examiner alleges that the record does not indicate in what way this region is correlated to HAL3 function or what other types of amino acid substitutions would be functionally tolerated at these specific location. As discussed above, the claims have been amended and remarks provided as to why a person skilled in the art would consider preferentially the C-terminal half without the acidic region of yeast HAL3 as the part conferring salt tolerance. A person skilled in the art would also appreciate that within this C-terminal half, the conserved residues are the first candidates for being responsible for conferring salt tolerance.

A determination of undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or it the

specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Jackson*, 217 USPQ 804, 807-808 (Bd. App. 1982). The present application clearly teaches a method of obtaining the presently claimed nucleic acid molecules, host cells, vectors, transgenic plants comprising the foregoing, as well as methods for producing a cell cycle interacting protein. For example, a two-hybrid screening procedure is provided in Example 1 which identifies cell cycle interacting proteins which bind to a CDK having a PPTALRE cyclin binding motif as presently recited in the claims. *See* Example 4, page 72, where the Vb89 clone interacts with CDC2bAt, but not CDC2aAt. Next, based on homology to the yeast *HAL3* (and *SIS2*) gene isolated in yeast, the specification teaches that the nucleotides sequence encoding SEQ ID NO:8 is a halotolerant gene. The specification further teaches on page 7 of the application that VB89 comprises the amino acid sequence set forth in SEQ ID NO:8 and is encoded by the isolated nucleotide sequence set forth in SEQ ID NO:7. Pages 16-19 disclose the use of computer programs available to help in identifying if a clone interacting with CDC2bAt but not CDC2aAt is in fact a halotolerant gene based on sequence homologies. Further, hybridization experiments for use in identifying other plant halotolerant genes are provided at page 13 of the present invention. *See also* page 17-19 under the heading "Identifying derivative, variants and homologs of the cell cycle interacting proteins of the invention."

Still further, practical guidance for transformation of plants and for testing the expression of the transgene is given on page 38, last paragraph to page 42, line 4. Example 4 (page 72, third to last line to page 73, line 2) lists a number of suitable promoters for controlling expression of the HAL 3 encoding DNA sequence, and a more elaborate list of suitable promoters for controlling expression of the HAL3 encoding DNA sequence may be found on page 35 to 37.

From page 93, last line to page 96, line 2, general protocols for plant cultivation are provided. A person skilled in the art would know how to subject plants to salt stress and how to evaluate the effects of salt on plant growth, for example by supplying 25, 50 or 100 mM salt to the growth medium and by measuring the effects on root or shoot length, or on root and/or shoot biomass. These types of experiments are routine and commonly known in the art.

Moreover, one skilled in the art would be able to apply the procedure outlined in Example 5 (page 79,line 11, to page 85, first paragraph and Table 5) to find other HAL3 homologues that interact with a CDK having a PPTALRE cyclin binding motif, thereby using the conserved regions of the known HAL3 proteins as a source sequence for primer design. If in the first instance only partial sequences were isolated, the strategy outlined in Example 8 could be used to isolate full-length clones.

Further, as submitted previously, one skilled in the art, comparing the highly significant homology of Vb89 (SEQ ID NO:8) to the publicly available HAL3 amino acid sequence from *S. cerevisiae* referenced in the present application, could fairly deduce the strong homology between the region of amino acids 96-118 of SEQ ID NO:8 and the corresponding region of *S. cerevisiae* HAL3. A determination of undue experimentation is not merely quantitative, since a *considerable* amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Jackson,*217 USPQ 804, 807-808 (Bd. App. 1982). Thus, although it might have taken a considerable amount of experimentation at the time of filing the present application to identify derivatives and homologs to Applicants' halotolerant protein comprising the amino acid sequence set forth in SEQ ID NO:8, such experimentation would not have been considered undue.

On page 8 of the Office Action, the Examiner alleges that the claimed invention is not enabled because the polypeptide that exhibits homology to HAL3 lacks the HAL3 region required for salt tolerance activity as could be derived from Ferrando et al. However, as discussed above, the later publications by Rodriguez et al. and Espinosa-Ruiz et al., demonstrated that the assumption of Ferrando et al. was indeed wrong.

Also on page 8, the Examiner alleges that the claimed invention is not enabled because the effect of making amino acid substitutions in a conserved region of a polypeptide is unpredictable, thereby citing Rhoads et al. (J. Biol. Chem. 273, 30750-30756). However the paper by Rhoads et al. does not teach anything about HAL3 and the substitution made by Rhoads et al. from Cys (a hydrophilic and polar amino acid) to Ala (a hydrophobic non-polar residue) is not a conserved substitution and thus likely to be unpredictable. One skilled in the art would know to make conserved substitutions in areas showing great homology to other halotolerant proteins, such as in amino acids 96to 118 of SEQ ID NO:8. Accordingly, based on the foregoing remarks and amendments to the claims, withdrawal of the rejection of claims 1, 5-10, 13-23 and 50 under the enablement provision of 35 U.S.C.§ 112, first paragraph is warranted.

Claims 1, 5-10, 13-23, and 50 remain rejected under 35 U.S.C.§ 101, as allegedly not supported by either a specific and substantial asserted utility or a well established utility, for the reasons set forth in the previous office action. According to the Examiner, "[n]either Applicants nor the prior art provide any evidence, other than the conservation of sequence itself, that the conserved structural feature corresponding to the functional HAL3 sequence recited in currently amended claim 1 is sufficient to impart salt tolerance activity on SEQ ID NO:8." The Examiner again relies on the finding by Ferrando et al. that amino acids outside the region of HAL3 corresponding to residues 96-118 of SEQ ID NO:8

are required for HAL3 salt tolerance activity. As described above, the findings by Ferrando et al. have been shown by others to be inaccurate.

The specification is replete with teachings of the presently claimed invention being useful for conferring salt tolerance in plants. *See e.g.,* page 34, lines 7-10 where it is stated: "overproduction of the cell cycle interacting protein of the present invention enhances growth and results in cell division to be less sensitive to an arrest caused by environmental stress such as salt, nutrient deprivation, drought, chilling and the like." Similar statements may be found on page 49, lines 9-11, page 61, lines 9-20, page 62, lines 5-8 and line 29 to page 63, line 5. Thus, apparently, the Examiner believes that the assertion in the specification for a specific and substantial utility would not be considered credible by a person of ordinary skill in the art. *See* US PTO's Utility Guidelines, Fed. Reg. 66(4):1092-1099 (Friday, January 5, 2001). As provided by the Guidelines, "credibility is assessed from the perspective of one or ordinary skill in the art in view of the disclosures and any other evidence of record (*e.g.,* test data, affidavits or declarations from experts in the art, patents or printed publications) that is probative of applicant's assertions. An applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement.

With respect to the Examiner's comments that "[n]either Applicants nor the prior art provide any evidence, other than conservation of sequence itself, that the conserved structural feature corresponding to the functional HAL3 sequence recited in currently amended claim 1 is sufficient to impart salt tolerance activity on SEQ ID NO:8", Applicants submit the following remarks. As previously discussed in the submission under 37 C.F.R.§ 1.114, filed as part of the RCE in the above-identified application on March 5, 2004, Applicant's VB89 halotolerant protein (SEQ ID NO:8) and HAL3 from *Saccharomyces cerevisiae* (publicly available prior to the original filing date of this application) share a mere 13% amino acid sequence identity and a 21.7% amino acid sequence similarity. *See* Exhibit B of March 5,

2004 submission. The most conserved region between the two proteins correlates to amino acids 96 to 118 of SEQ ID NO:8, the area of identity being less than the 80% identity as presently claimed. Yet Applicants accurately described the utility of the presently claimed invention, as later publications have confirmed. Specifically, as the publications provided herewith as Exhibits 1-4 clearly illustrate, although the sequence of SIS2 and YKL088 are only 23.6% identical, YKL088 is able to complement a salt sensitive yeast strain in the same way as SIS2. Similarly, although the sequence identities of AtHAL3a and AtHAL3b with SIS2 are lower compared to YKL088s with SIS2, the sequence conservation is in the same region of the proteins. Thus, the findings of those of skill in the art, provided herewith as Exhibits 1-4, offer objective evidence supporting the utility of the present invention. *See* Gould v. Quigg, 822 F.2d 1079, 3 USPQ 2d 1302 (Fed. Cir. 1987)(later dated publication may be used as evidence of the level of ordinary skill in the art at the time of the application and as evidence that disclosed device would have been operative).

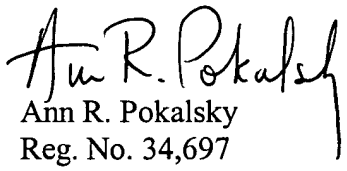
Applicants have therefore established a probative relation between the submitted evidence of Exhibits 1-4 and the originally disclosed properties of the claimed invention. Withdrawal of the rejection of Claims 1, 5-10, 13-23 and 50 under 35 U.S.C.§ 101 is therefore warranted.

Claim 5 has been rejected under 35 U.S.C. 102(b) as allegedly anticipated by Ferrando et al. (1995) *Molecular and Cellular Biology* 15 (10): 5470-5481. Ferrando et al. has been cited for teaching an isolated nucleic acid molecule comprising a nucleotide sequence encoding a 652 amino acid HAL3 sequence obtained from *Saccharomyces cerevisiae*. As presently amended, claim 5 recites: "[a]n isolated nucleic acid molecule of at least 15 nucleotides in length from a plant halotolerant gene, wherein the isolated nucleic acid molecule hybridizes specifically with a DNA molecule of claim 1 or with a complementary strand thereof." Since the presently amended claim 5 recites a nucleic acid molecule

isolated from a plant halotolerant gene, claim 5 is distinguished from the disclosure of Ferrando et al. Withdrawal of the rejection of claim 5 under 35 U.S.C. 102(b) is therefore warranted.

In view of the foregoing remarks, Exhibits 1-4 and amendments to the claims, it is firmly believed that the present application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



Ann R. Pokalsky
Reg. No. 34,697
Attorney for Applicants

DILWORTH & BARRESE
333 Earle Ovington Blvd.
Uniondale, NY 11553
(516) 228-8484

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CtCdc55p and CtHal3p: Two Putative Regulatory Proteins from *Candida tropicalis* with Long Acidic Domains

PEDRO L. RODRIGUEZ†, RASHID ALI‡ AND RAMON SERRANO*

Instituto de Biologia Molecular y Celular de Plantas, Universidad Politecnica de Valencia-C.S.I.C., Camino de Vera s/n, 46022 Valencia, Spain

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The salt-tolerance gene *HAL3* from *Saccharomyces cerevisiae* encodes a novel regulatory protein (Hal3p) which modulates the expression of the *ENA1* sodium-extrusion ATPase (Ferrando *et al.*, *Mol. Cell. Biol.* vol. 15, 1995, pp. 5470–5481). Hal3p contains an essential acidic domain rich in aspartates at its carboxyl terminus. We have isolated two cross-hybridizing genes from a genomic library of *Candida tropicalis*. One of the genes (*CtHAL3*) is a true homolog of *HAL3* and it partially complements the salt sensitivity of a *S. cerevisiae hal3* mutant. The activity of *CtHAL3* was equivalent to that of an open reading frame (YKL088w) identified by genome sequencing of *S. cerevisiae* and with homology to *HAL3*. The other cross-hybridizing gene (*CtCDC55*) is a *CDC55* homolog, encoding a protein with an internal acidic domain not present in the *S. cerevisiae CDC55* product. Cdc55p is a regulatory subunit of protein phosphatase 2A and *CtCDC55* complements the cold sensitivity of a *S. cerevisiae cdc55* mutant. The presence of acidic domains in different putative regulatory proteins may suggest a role for this type of domain in molecular interactions. Sequences have been deposited in the EMBL data library under Accession Numbers X88899 (*CtCDC55*) and X88900 (*CtHAL3*).

KEY WORDS — *Candida tropicalis*; *CDC55*; *HAL3*; protein phosphatase; acidic domain

INTRODUCTION

A novel regulatory protein from *Saccharomyces cerevisiae*, Sis2p/Hal3p (in the following referred to as Hal3p), has recently been identified which contains a long acidic domain at its carboxyl terminus (Di Como *et al.*, 1995; Ferrando *et al.*, 1995). Hal3p is involved in a signal transduction pathway required for maximum expression of G1 cyclins (Di Como *et al.*, 1995) and of the *ENA1* gene, a major determinant of salt tolerance encoding a putative sodium-pumping ATPase (Ferrando *et al.*, 1995). The long acidic domain is essential for Hal3p function, suggesting that it could participate in regulatory interactions with proteins or small molecules. The subcellular localization of Hal3p is controversial and both a nuclear (Di Como *et al.*, 1995) and a cytosolic (Ferrando *et al.*, 1995) location have been reported. The Hal3p pathway seems to act in parallel to two protein phosphatases modulating gene expression: the Sit4p protein phosphatase in the case of G1 cyclins (Fernandez-Sarabia *et al.*, 1992; Di Como *et al.*, 1995) and calcineurin (a calcium and calmodulin-activated protein phosphatase) in the case of *ENA1* (Mendoza *et al.*, 1994; Ferrando *et al.*, 1995). Therefore, it has been suggested that Hal3p could participate in a novel phosphatase pathway (Ferrando *et al.*, 1995).

In order to explore the general occurrence of the Hal3p regulatory pathway, we have isolated two *HAL3*-related genes from *Candida tropicalis*. The first of them is a true homolog of *HAL3* and the second one, although containing a long acidic domain responsible for the cross-hybridization signal, is homologous to *S. cerevisiae CDC55*, a regulatory subunit of protein phosphatase 2A.

*Corresponding author.
†Present address: Institute für Pflanzenwissenschaften, ETH, Universitätstrasse 2, CH-8092 Zürich, Switzerland.
‡Present address: National Institute for Biotechnology and Genetic Engineering, Jhang Road, PO Box 577, Faisalabad, Pakistan.

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Table 1. Yeast strains used in this study.

Strain	Genotype	Source
RS16 (*S. cerevisiae*)	*MAT***a** *ura3-251,328,372 leu2-3,112*	Gaxiola *et al.* (1992)
RS48 (*S. cerevisiae*)	RS16 *hal3::LEU2*	Ferrando *et al.* (1995)
AHY80 (*S. cerevisiae*)	*MAT***a** *cdc55::LEU2 leu2 his3*	Healy *et al.* (1991)
AHY20(*S. cerevisiae*)	*MAT***a** *ura3*	Healy *et al.* (1991)
NCYC2512 (*C. tropicalis*)	Wild type	R. Ali (this work)

The possible function of acidic domains in protein phosphatase-mediated signal transduction is suggested.

MATERIALS AND METHODS

Yeast strains and growth media

The *S. cerevisiae* and *C. tropicalis* strains used in this study are listed in Table 1. Cells were routinely grown in YPD medium (1% yeast extract, 2% Bacto peptone and 2% glucose). Transformants (Ito *et al.*, 1983) were selected by plating on minimal medium (SD) containing 2% glucose, 0·7% yeast nitrogen base without amino acids (Difco), 50 mM-MES adjusted to pH 6·0 with Tris and supplemented with the indicated requirements (leucine 100 μg/ml, uracil 30 μg/ml or histidine 30 μg/ml). YPD and SD were solidified with 2% agar. Growth on high salt medium was tested on YPD supplemented with 1 M-NaCl by a drop assay (Gaxiola *et al.*, 1992; Ferrando *et al.*, 1995).

Construction and screening of a genomic DNA library from C. tropicalis *in YEP351*

Genomic DNA from *C. tropicalis* strain NCYC2512 was prepared and a library for cloning in *S. cerevisiae* constructed basically as described by Rose (1987). The genomic DNA was partially digested with *Sau*3A and fragments between 7–8 kb in size were selected by agarose electrophoresis, freeze-squeeze purified (Tautz and Renz, 1983) and ligated to *Bam*HI-digested, alkaline phosphatase-treated YEp351, a shuttle plasmid derived from the 2 μ circle and with the *LEU2* gene as marker (Hill *et al.*, 1986). After electroporation-mediated transformation (Dower *et al.*, 1988) of *Escherichia coli* strain WM1100 (a *recA* derivative of MC1061; Miller, 1987), 50 000 ampicillin-resistant colonies were obtained, pooled and stored in 15% glycerol at −70°C. The complete reading frame of *HAL3* was amplified by polymerase chain reaction (PCR) as described (Ferrando *et al.*, 1995), labelled with ^{32}P by the random-priming method (Feinberg and Vogelstein, 1983) and used as a probe to screen the library by colony hybridization (Hanahan and Meselson, 1980). High stringency conditions were employed for hybridization (65°C, 0·8 M-NaCl ionic strength equivalent) and washes (65°C, 80 mM-NaCl ionic strength equivalent). Two colonies, out of 10 000 tested, cross-hybridized with *HAL3*. Plasmid DNA was isolated and cross-hybridizing restriction fragments were subcloned into pBluescript (Stratagene). Unidirectional nested deletions were generated with exonuclease III and S1 nuclease (Henikoff, 1984) according to the 'Erase-a-Base' system of Promega (Madison, Wisconsin). Sequencing was by the dideoxy method and T7 DNA polymerase (Tabor and Richardson, 1987) according to the Sequenase system of USB (Cleveland, Ohio).

Expression of CtCDC55 *in* S. cerevisiae

The *C. tropicalis CDC55* (*CtCDC55*) gene was expressed in *S. cerevisiae* from its own promoter. The 3 kb *Xho*I-*Bgl*II fragment hybridizing with *HAL3* (Figure 1) was subcloned into pBluescript KS (Stratagene, La Jolla, California) digested with *Xho*I and *Bam*HI. *C. tropicalis* DNA was liberated from the resulting plasmid as a *Xho*I-*Sac*I fragment and subcloned into yeast centromeric plasmid pUN90 (*HIS3* marker; Elledge and Davis, 1988) digested with *Sal*I and *Sac*I to produce pUN90-*CtCDC55*. Both pUN90 and pUN90-*CtCDC55* were transformed (Ito *et al.*, 1983) into yeast strain AHY80 (*cdc55 his3*; Healy *et al.*, 1991) to test for complementation of the *cdc55* mutation.

Expression of CtHAL3 *in* S. cerevisiae

The *C. tropicalis HAL3* gene (*CtHAL3*) was expressed in *S. cerevisiae* from its own promoter. The 3 kb *Eco*RI fragment hybridizing with *HAL3* (Figure 1) was subcloned into YEp352, a shuttle

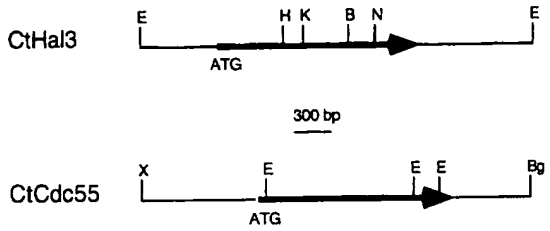

Figure 1. Restriction map of two genomic regions of *C. tropicalis* containing ORFs (arrows) with long acidic domains. *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N) and *Xho*I (X) sites are indicated.

plasmid derived from the 2 μ circle and with *URA3* as marker (Hill *et al.*, 1986), to produce YEp352-*CtHAL3*. Both YEp352 and YEp352-*CtHAL3* were transformed (Ito *et al.*, 1983) into yeast strains RS16 (Hal3$^+$) and RS48 (hal3) to test for salt tolerance. As a comparison, plasmid YEp352-*ScHAL3* (Ferrando *et al.*, 1995) was also transformed into the same strains.

CtHAL3 was also expressed in *S. cerevisiae* from the strong *PMA1* promoter (Serrano and Villalba, 1995). The complete reading frame of *CtHAL3* was PCR-amplified with upstream primer 5'-GGCCGGCTCGAGATGCCTTCTGATAAGG ATATT and downstream primer 5'-GGGC CCCTCGAGTCAAAGGTTAGTAGTTTCATC (both introduce a *Xho*I site, underlined). After digestion with *Xho*I, the 1·6 kb PCR fragment was subcloned with the right orientation into the *Xho*I site of yeast expression plasmid pRS699 (Serrano and Villalba, 1995), to produce pRS699-*CtHAL3*. This plasmid was transformed into yeast strains as described above.

Cloning and expression in S. cerevisiae *of YKL088w*

The open reading frame (ORF) YKL088w of yeast chromosome XI (Dujon *et al.*, 1994) has significant homology to *HAL3* (Ferrando *et al.*, 1995). It was cloned by performing PCR on *S. cerevisiae* genomic DNA (Rose, 1987). The upstream primer was 5'-CCGGCCCTCGAG ATGACGGATGAAAAAGTGAAC and the downstream primer 5'-CGCGCGCTCGAGTT AAACTTCGGTTTTCACGTC (both introduce a *Xho*I site, underlined). Amplification was performed by 30 cycles of incubations at 94°C for 1 min, 50°C for 1 min and 72°C for 3 min. After digestion with *Xho*I, the PCR product (1·7 kb) was subcloned into the *Xho*I site of yeast expression plasmid pRS699 (Serrano and Villalba, 1995). A plasmid (pRS699-YLK088w) was selected with the YKL088w ORF under control of the constitutive *PMA1* promoter and transformed into yeast strains as described above. A positive control plasmid (pRS699-*ScHAL3*) was constructed with the ORF of *S. cerevisiae HAL3* under control of the *PMA1* promoter. The construct was made as described above for YKL088w. The upstream PCR primer as 5'-GGCCGGCTCGAGATGAC TGCCGTCGCCTCTACT and the downstream PCR primer 5'-GGGCCCCTCGAGTTATTGA TGCTTATCTATTAT (both introduce a *Xho*I site, underlined).

RESULTS AND DISCUSSION

Cloning and identification of CtHAL3 *and* CtCDC55

Approximately 10 000 colonies from the *C. tropicalis* genomic library were screened by their ability to cross-hybridize to a *S. cerevisiae HAL3* probe. Two cross-hybridizing clones were identified (Figure 1). One of them contained a 3 kb *Eco*RI cross-hybridizing fragment. Sequencing revealed a novel *C. tropicalis* gene, *CtHAL3*, which encoded a protein of 531 amino acids with significant homologies to Hal3p at its second half (Figure 2). Also included in Figure 2 is the ORF YKL088w (571 amino acids) of *S. cerevisiae* chromosome XI (Dujon *et al.*, 1994), which also has significant homology to *HAL3* at its second half (Ferrando *et al.*, 1995). Altogether these three genes define a novel family of proteins containing a conserved domain of about 200 amino acids at the carboxyl-terminal half. The most conserved motifs within this domain are the polar sequence ELR(R,K)WAD, the cysteine region (G(I,L) C(N,D)NLLT, the glycine-rich loop GDIG (L,K)GG and an acidic tail with 40–50 aspartates and glutamates (Figure 2). There is no significant homology within the amino-terminal half of the proteins.

Plasmid DNA corresponding to the second clone contained a 3 kb *Xho*I-*Bgl*II cross-hybridizing fragment (Figure 1). Sequencing revealed a new *C. tropicalis* gene, *CtCDC55*, which encodes a protein of 509 amino acids highly homologous to the product of the *CDC55* gene of *S. cerevisiae* (Figure 3; Healy *et al.*, 1991). *CtCDC55* contains an internal acidic domain responsible for the cross-hybridization signal to

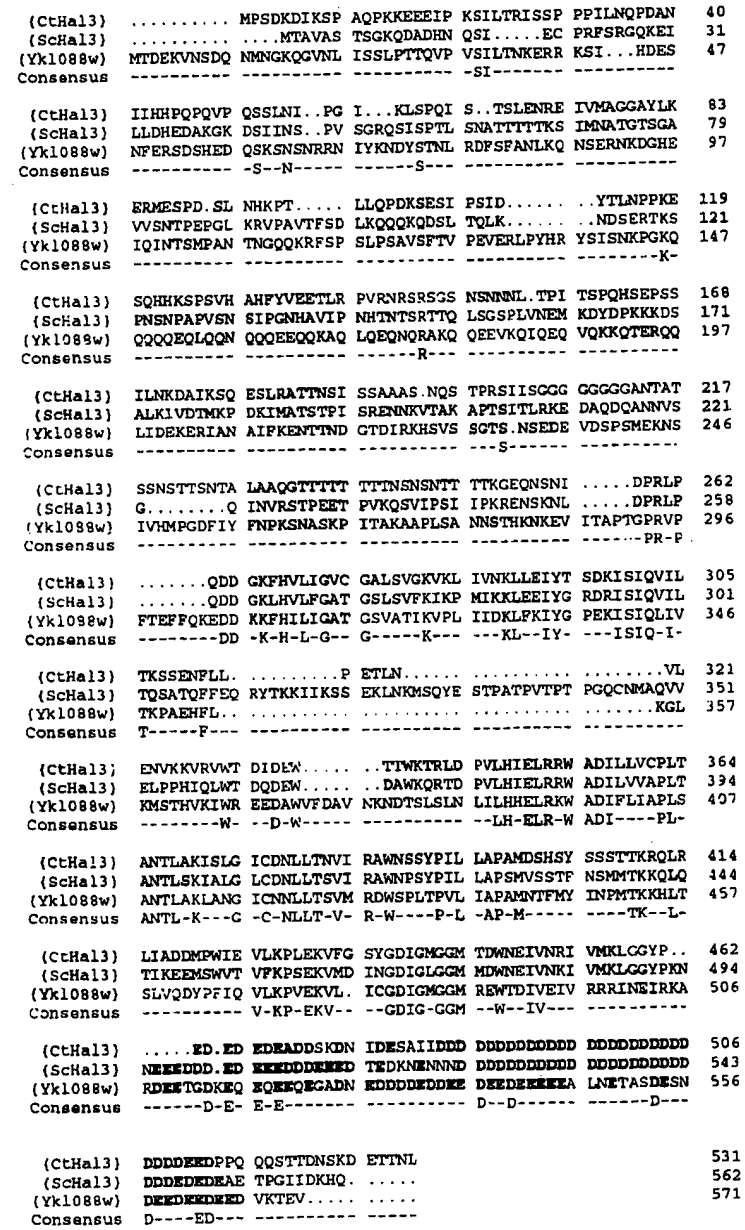

Figure 2. Comparison of the *C. tropicalis HAL3* protein (CtHal3) with the *S. cerevisiae HAL3* protein (ScHal3) and with the ORF YKL088w predicted protein (YKL088w). DNA and deduced protein sequences were analysed using the GCG software package.

HAL3 and which is not present in *CDC55*. Outside this acidic domain, *CtCDC55* has no significant homology to *HAL3*.

S. cerevisiae CDC55 encodes the PR55 subunit (or regulatory subunit B) of protein phosphatase 2A, one of the major serine/threonine-specific phosphatases (Shenolikar, 1994). The core enzyme consists of a 36 kDa catalytic subunit and a 65 kDa regulatory subunit (PR65 or subunit A). It associates with a third, variable regulatory subunit of either 55 (PR55 or subunit B), 72 (PR72 or subunit C) or 130 (PR130 or subunit C') kDa.

```
(DmCdc55)  MGRWGRQSPV LEPPDPQMQT TPPPPTLPPR TFMRQSSITK IGNMLNTAIN   50
(HsCdc55)  .......... .......... .......... .......... ..........
(CtCdc55)  .......... .......... .......... .......... ..........
(ScCdc55)  .......... .......... .......... .......... ..........

(DmCdc55)  INGAKKPASN GEASWCFSQI KGALDD..DV TDADIISCVE FNHDGELLAT   98
(HsCdc55)  MAGA...GGG NDIQWCFSQV KGAVDD..DV AEADIISTVE FNHSGELLAT   45
(CtCdc55)  .......... ..MNLDFSQC FGDKGDIENI TEADIISTVE FDHTGDFLAT   38
(ScCdc55)  .......MAQ NNFDFKFSQC FGDKADIV.V TEADLITAVE FDYTGNYLAT   42
Consensus  ---------- ------FSQ- -G---D---- --AD-I--VE F---G--LAT

(DmCdc55)  GDKGGRVVIF QRDPASKAAN PRRGEYNVYS TFQSHEPEFD YLKSLEIEEK  148
(HsCdc55)  GDKGGRVVIF QQEQENKIQS HSRGEYNVYS TFQSHEPEFD YLKSLEIEEK   95
(CtCdc55)  GDKGGRVVLF ERNQSKKKQS ...CEYKFFT EFQSHDAEFD YLKSLEIEEK   85
(ScCdc55)  GDKGGRVVLF ERSNSRH... ...CEYKFLT EFQSHDAEFD YLKSLEIEEK   86
Consensus  GDKGGRVV-F ---------- ----EY---- -FQSH--EFD YLKSLEIEEK

(DmCdc55)  INKIRWLQQK NPVHFLLSTN DKTVKLWKVS ERDKSFGGYN TKEE......  192
(HsCdc55)  INKIRWLPQK NAAQFLLSTN DKTIKLWKIS ERDKRPEGYN LKEE......  139
(CtCdc55)  INKIKWLKSA NDSLCLLSTN DKTIKLWKIQ ERQIKLVSEN NLNGLNHLPS  135
(ScCdc55)  INEIKWLRPT QRSHFLLSTN DKTIKLWKVY EKNIKLVSQN NLTEGVTFAK  136
Consensus  IN-I-WL--- -----LLSTN DKT-KLWK-- E--------N ----------

(DmCdc55)  .......... NGLIRDPQNV TALRVPSVKQ IPLLVEASPR RTFANAHTYH  232
(HsCdc55)  .......... DGRYRDPTTV TTLRVPVFRP MDLMVEASPR RIFANAHTYH  179
(CtCdc55)  SN........ .......IGI ESLKLPQLQL HDKLISAQPK KIYANAHAYH  170
(ScCdc55)  KGKPDNHNSR GGSVRAVLSL QSLKLPQLSQ HDKIIAATPK RIYSNAHTYH  186
Consensus  ---------- ---------- --L--P---- ------A-P- ----NAH-YH

(DmCdc55)  INSISVNSDQ ETFLSADDLR INLWHLEVVN QSYNIVDIKP TNMEELTEVI  282
(HsCdc55)  INSISINSDY ETYLSADDLR INLWHLEITD RSFNIVDIKP ANMEELTEVI  229
(CtCdc55)  INSISVNSDQ ETYLSADDLR INLWNLGIAD QSFNIVDIKP ANMEELTEVI  220
(ScCdc55)  INSISLNSDQ ETFLSADDLR INLWNLDIPD QSFNIVDIKP TNMEELTEVI  236
Consensus  INSIS-NSD- ET-LSADDLR INLW-L---- -S-NIVDIKP -NMEELTEVI

(DmCdc55)  TAAEFHPTEC NVFVYSSSKG TIRLCDMRSA ALCDRHSKQF EEPENPTNRS  332
(HsCdc55)  TAAEFHPNSC NTFVYSSSKG TIRLCDMRAS ALCDRHSKLF EEPEDPSNRS  279
(CtCdc55)  TSAEFHPLQC NLFMYSSSKG TIKLSDMRSN SLCDSHAKIF EEYLDPSSHN  270
(ScCdc55)  TSAEFHPQEC NLFMYSSSKG TIKLCDMRQN SLCDNKTKTF EEYLDPINHN  286
Consensus  T-AEFHP--C N-F-YSSSKG TI-L-DMR-- -LCD---K-F EE---P----

(DmCdc55)  FFSEIISSIS DVKLSNSGRY MISRDYLSIK VWDLHMETKP IETYPVHEYL  382
(HsCdc55)  FFSEIISSIS DVKFSHSGRY MMTRDYLSVK IWDLNMENRP VETYQVHEYL  329
(CtCdc55)  FFTEITSSIS DVKFSHDGRY IASRDYMTVK IWDLAMENKP IKTIDVHEHL  320
(ScCdc55)  FFTEITSSIS DIKFSPNGRY IASRDYLTVK IWDVNMDNKP LKTINIHEQL  336
Consensus  FF-EI-SSIS D-K-S--GRY ---RDY---K -WD--M---P --T---HE-L

(DmCdc55)  RAKLCSLYEN DCIFDKFECC WNGKDSSIMT GSYNNFFRVF ..........  422
(HsCdc55)  RSKLCSLYEN DCIFDKFECC WNGSDSVVMT GSYNNFFRMF ..........  369
(CtCdc55)  RERLCDTYEN DAIFDKFEVQ FGGDNKSVMT GSYNNQFVIY PNAVNTGNDD  370
(ScCdc55)  KERLSDTYEN DAIFDKFEVN FSGDSSSVMT GSYNNNFMIY PNVVTSGDND  386
Consensus  ---L---YEN D-IFDKFE-- --G-----MT GSYNN-F--- ----------

(DmCdc55)  .......... .DRNSKK... .......... .......... ..........  428
(HsCdc55)  .......... .DRNTKR... .......... .......... ..........  375
(CtCdc55)  KPKFKSAFKN SSKRSKKNGF STRTTDDDDD DDDDDDDEEA DDEFDEEVPA  420
(ScCdc55)  NGIVKTFDEH NAPNSNSNKN IHNSIQNKDS SSSGNSHKRR SNGRNTGMVG  436
Consensus  ---------- ---------- ---------- ---------- ----------

(DmCdc55)  .......... .......... .....DVTLE ASRDIIKPK. .TVLKPRKVC  451
(HsCdc55)  .......... .......... .....DITLE ASRENNKPR. .TVLKPRKVC  397
(CtCdc55)  TKNSPGSQLE DDD..... ..EQEEIILQ ADKSAFKSKK SGQHPMRRRM  461
(ScCdc55)  SSNSSRSSIA GGEGANSEDS GTEMNEIVLQ ADKTAFRNKR YGSLAQR...  483
Consensus  ---------- ---------- --------L- A--------- ------R---

(DmCdc55)  TGGKRKKDEI SVDCLDFNKK ILHTAWHPEE NIIAVAATNN LFIFQDKF.   499
(HsCdc55)  ASGKRKKDEI SVDSLDFNKK ILHTAWHPKE NIIAVATTNN LYIFQDKVN  447
(CtCdc55)  TSGVGSNLGR EFDDVDFKKS ILHLSWHPRE NSVAIAATNN LYIFSTL..  508
(ScCdc55)  .SARNKDWG. ..DDIDFKKN NLHFSWHPRE NSIAVAATNN LFIFSAL..  526
Consensus  ---------- --D--DF-K- -LH--WHP-E N--A-A-TNN L-IF-----
```

Figure 3. Comparison of the *C. tropicalis CDC55* protein (CtCdc55) with the homolog B subunits of protein phosphatase 2A from *Drosophila melanogaster* (DmCdc55; Mayer-Jaekel *et al.*, 1993), human α isoform (HsCdc55; Mayer *et al.*, 1991) and *S. cerevisiae* (ScCdc55; Healy *et al.*, 1991). The acidic domain of CtCDC55p is shown in bold face. DNA and deduced protein sequences were analysed using the GCG software package.

All subunits are conserved from yeast to man (Mayer *et al.*, 1991; Mayer-Jaeckel *et al.*, 1993; Mayer-Jaekel and Hemmings, 1994). Comparison of some PR55 proteins shows this high conservation (Figure 3). However, a distinctive feature of the product of the *CtCDC55* gene is the presence of an acidic domain. As compared to animal PR55s, both the *C. tropicalis* and *S. cerevisiae* homologs contain an insertion of about 70 amino acids, which in *C. tropicalis*, but not in *S. cerevisiae*, includes 18 aspartates and 8 glutamates (in bold in Figure 3). This acidic domain is 70 amino acids away from the carboxyl terminus.

Southern blot analysis of genomic DNA (results not shown) confirmed the presence of single *CtHAL3* and *CtCDC55* genes in the *C. tropicalis* genome. Accession numbers in EMBL nucleotide sequence data base are X88900 and X88899 for *CtHAL3* and *CtCDC55*, respectively.

CtHAL3p and the predicted S. cerevisiae *YKL088w protein partially complement the salt sensitivity of a* hal3::LEU2 S. cerevisiae *strain*

Disruption of *HAL3* in *S. cerevisiae* results in salt sensitivity (Ferrando *et al.*, 1995). In order to test whether *CtHAL3* and YKL088w (see above) are functional homologs to *HAL3*, we have tried to complement this *hal3* phenotype by expression of the genes (Figure 4). Both *CtHAL3* (panel 3) and *YKL088w* (panel 7) complemented the salt sensitivity of a *hal3* mutant, suggesting that these genes encode proteins with similar activities to *S. cerevisiae* Hal3p. Overexpression of *S. cerevisiae HAL3* with the same plasmids results in higher salt tolerance (panels 4 and 8). However, as the salt tolerance effect of *HAL3* is dose dependent (Ferrando *et al.*, 1995), the relative activities of the different proteins cannot be compared without information on their expression levels. Expression of *CtHAL3* from the strong *PMA1* promoter (pRS699-*CtHAL3*, see Materials and Methods) did not improve the salt tolerance effect of the gene expressed from its own promoter (data not shown).

CtCDC55 *encodes a functional homolog of* S. cerevisiae CDC55

S. cerevisiae cdc55 mutants display a cold-sensitive phenotype characterized by morphogenetic defects at low temperature (Healy *et al.*, 1991). To test whether *CtCDC55* is a functional homolog of *CDC55*, a complementation assay

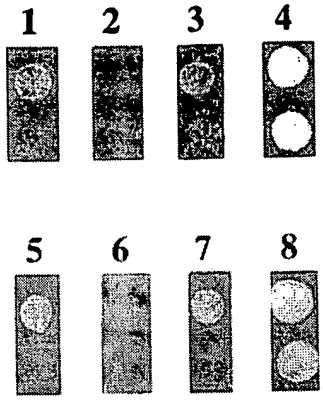

Figure 4. Complementation assay of the *S. cerevisiae hal3* mutant. Drops (3 μl) of 1/10 (top) and 1/100 (bottom) dilutions of saturated cultures were spotted onto YPD plates supplemented with 1 M-NaCl and incubated at 28°C for 5 days. (1) Wild-type strain RS16 (*HAL3*) transformed with control plasmid YEp352; (2–4) strain RS48 (*hal3::LEU2*) transformed with plasmids YEp352 (2), YEp352-*CtHAL3* (3) or YEp352-*ScHAL3* (4); (5) strain RS16 (*HAL3*) transformed with control plasmid pRS699; (6–8) strain RS48 (*hal3::LEU2*) transformed with plasmids pRS699 (6), pRS699-YLK088w (7) or pRS699-*ScHAL3* (8). Identical results were obtained with three different transformants from every plasmid.

was carried out. *S. cerevisiae* strain AHY80 (*cdc55::LEU2* disruption mutant) was transformed with a pUN90 centromeric vector containing the 3 kb *Xho*I/*Bgl*II *CtCDC55* fragment and growth was tested at 28°C and 14°C (Figure 5A). The *cdc55* mutant (columns 1 and 3) shows a growth delay at low temperature as compared to wild type (column 2). However, after being transformed with the *CtCDC55* gene, it was able to grow at 14°C as wild type. The aberrant morphology developed at low temperature by the *cdc55* mutant (Figure 5B, panel 3) was also reverted by the *CtCDC55* gene (Figure 5B, panel 4). This demonstrates that *CtCDC55* is a true functional homolog of *S. cerevisiae CDC55*.

Long acidic domains in putative regulatory proteins

S. cerevisiae Hal3p (Ferrando *et al.*, 1995), CtHal3p and CtCdc55p (present work) are examples of putative regulatory proteins with long acidic domains. Domains with more than 20 glutamates and/or aspartates have been identified in nuclear proteins such as centromere protein CENP-B, non-histone proteins HMG-1,2, nucleolin and nucloplasmin (Earnshaw, 1987). These domains are much more acidic than activator

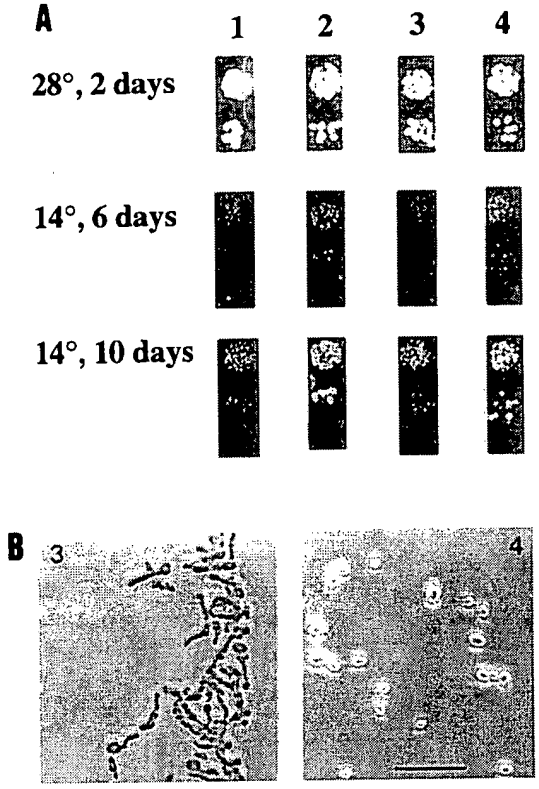

Figure 5. Complementation assay of the *S. cerevisiae cdc55* mutant. (A) Drops (3 µl) of 1/10 (top) and 1/100 (bottom) dilutions of saturated cultures were spotted onto YPD plates and incubated at 28°C or 14°C for the days indicated. (1) Strain AHY80 (*cdc55:LEU2*); (2) wild-type strain AHY20 (*CDC55*); (3) strain AHY80 transformed with control plasmid pUN90; (4) strain AHY80 transformed with pUN90-*CtCDC55*. Identical results were obtained with three different transformants from every plasmid. (B) Morphology of strain AHY80 (*cdc55::LEU2*) transformed with pUN90 or pUN90-*CtCDC55*. Phase contrast photomicrographs (Nikon104 microscope) were taken of the colonies shown in panel 1, lanes 3 and 4, after 10 days at 14°C. Magnification bar corresponds to 35 µm.

domains of transcription factors such as Gal4p and Gcn4p. The UBF transcription factor has a long acidic tail which may participate in nucleolar targeting (Maeda *et al.*, 1992) and a general role for acidic domains in unfolding chromatin structure by electrostatic 'capture' of histones has been proposed (Earnshaw, 1987). Calsequestrin and calreticulin, calcium-binding proteins of animal (Fliegel *et al.*, 1987; Michalak *et al.*, 1992) and plant (Krause *et al.*, 1989; Menegazzi *et al.*, 1993) microsomes contain a long acidic tail involved in low-affinity calcium binding and in retention within the lumen of the endoplasmic reticulum (Michalak *et al.*, 1992). Therefore, acidic domains of nuclear and microsomal proteins may have multiple functions.

The acidic tail of *S. cerevisiae* Hal3p is essential for its salt tolerance activity (Ferrando *et al.*, 1995). Hal3p has no signal peptide and is not a microsomal protein but is probably located at the cytoplasm (Ferrando *et al.*, 1995) and/or nucleus (Di Como *et al.*, 1995). Calcium binding to Hal3p measured by a $^{45}Ca^{2+}$ overlay assay (Krause *et al.*, 1989) gave negative results (A. Ferrando and R. Serrano, unpublished observations) and genetic evidence indicates that Hal3p does not participate in the transduction of the salt stress signal mediated by calcineurin and which probably involves calcium changes (Marquez and Serrano, 1996). Therefore, a role of the acidic domain of Hal3p in protein–protein interactions seems more likely than calcium binding. In this respect it can be mentioned that the cytoplasmic Hsp90 chaperone contains an acidic region that is thought to interact with several steroid hormone receptors (Binart *et al.*, 1995).

Overexpression of *S. cerevisiae HAL3/SIS2* suppresses both the salt sensitivity conferred by lack of the protein phosphatase 2B calcineurin (Ferrando *et al.*, 1995) and the reduced expression of G1 cyclins conferred by lack of the protein phosphatase 2A Sit4p (Di Como *et al.*, 1995). Therefore, it has been proposed that Hal3p is a regulatory subunit of some unidentified protein phosphatase (Ferrando *et al.*, 1995). Its essential acidic tail could mediate binding to the catalytic subunit of the phosphatase. In this respect, CtCdc55p is the first example of a phosphatase regulatory subunit containing a long acidic domain. A plausible function for acidic domains in regulatory proteins is that they constitute one type of module for interactions between subunits of protein complexes. It could be predicted that an acidic module in one protein would have a matching basic module in the interacting protein. In this respect, the acidic N-terminus of immunophilin FKBP46 has been described to interact with basic nuclear protein TP2 (Alnemri *et al.*, 1994). The identification of the catalytic subunit of protein phosphatase 2A in *C. tropicalis* and of the proteins interacting with Hal3p could provide additional evidence for this hypothesis. It must be indicated, however, that Cdc55p from *S. cerevisiae* does not contain an acidic domain (Healy *et al.*, 1991) and therefore electrostatic interactions between

domains are not essential for regulation of protein phosphatase 2A.

In addition to protein phosphatase complexes, acidic domains could mediate interactions within other types of regulatory complexes such as those nucleated by protein kinases. A subfamily of plant protein kinases has been described which contain acidic tails and which are induced by osmotic and temperature stresses (Holappa and Walker-Simmons, 1995). It would be interesting to investigate the role of this acidic domain in mediating interactions of the catalytic subunit of protein kinases with other regulatory subunits.

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CtCdc55p and CtHal3p: Two Putative Regulatory Proteins from *Candida tropicalis* with Long Acidic Domains

PEDRO L. RODRIGUEZ†, RASHID ALI‡ AND RAMON SERRANO*

Instituto de Biologia Molecular y Celular de Plantas, Universidad Politecnica de Valencia-C.S.I.C., Camino de Vera s/n, 46022 Valencia, Spain

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The salt-tolerance gene *HAL3* from *Saccharomyces cerevisiae* encodes a novel regulatory protein (Hal3p) which modulates the expression of the *ENA1* sodium-extrusion ATPase (Ferrando *et al.*, *Mol. Cell. Biol.* vol. 15, 1995, pp. 5470–5481). Hal3p contains an essential acidic domain rich in aspartates at its carboxyl terminus. We have isolated two cross-hybridizing genes from a genomic library of *Candida tropicalis*. One of the genes (*CtHAL3*) is a true homolog of *HAL3* and it partially complements the salt sensitivity of a *S. cerevisiae hal3* mutant. The activity of *CtHAL3* was equivalent to that of an open reading frame (YKL088w) identified by genome sequencing of *S. cerevisiae* and with homology to *HAL3*. The other cross-hybridizing gene (*CtCDC55*) is a *CDC55* homolog, encoding a protein with an internal acidic domain not present in the *S. cerevisiae CDC55* product. Cdc55p is a regulatory subunit of protein phosphatase 2A and *CtCDC55* complements the cold sensitivity of a *S. cerevisiae cdc55* mutant. The presence of acidic domains in different putative regulatory proteins may suggest a role for this type of domain in molecular interactions. Sequences have been deposited in the EMBL data library under Accession Numbers X88899 (*CtCDC55*) and X88900 (*CtHAL3*).

KEY WORDS — *Candida tropicalis*: *CDC55*; *HAL3*; protein phosphatase; acidic domain

INTRODUCTION

A novel regulatory protein from *Saccharomyces cerevisiae*, Sis2p/Hal3p (in the following referred to as Hal3p), has recently been identified which contains a long acidic domain at its carboxyl terminus (Di Como *et al.*, 1995; Ferrando *et al.*, 1995). Hal3p is involved in a signal transduction pathway required for maximum expression of G1 cyclins (Di Como *et al.*, 1995) and of the *ENA1* gene, a major determinant of salt tolerance encoding a putative sodium-pumping ATPase (Ferrando *et al.*, 1995). The long acidic domain is essential for Hal3p function, suggesting that it could participate in regulatory interactions with proteins or small molecules. The subcellular localization of Hal3p is controversial and both a nuclear (Di Como *et al.*, 1995) and a cytosolic (Ferrando *et al.*, 1995) location have been reported. The Hal3p pathway seems to act in parallel to two protein phosphatases modulating gene expression: the Sit4p protein phosphatase in the case of G1 cyclins (Fernandez-Sarabia *et al.*, 1992; Di Como *et al.*, 1995) and calcineurin (a calcium and calmodulin-activated protein phosphatase) in the case of *ENA1* (Mendoza *et al.*, 1994; Ferrando *et al.*, 1995). Therefore, it has been suggested that Hal3p could participate in a novel phosphatase pathway (Ferrando *et al.*, 1995).

In order to explore the general occurrence of the Hal3p regulatory pathway, we have isolated two *HAL3*-related genes from *Candida tropicalis*. The first of them is a true homolog of *HAL3* and the second one, although containing a long acidic domain responsible for the cross-hybridization signal, is homologous to *S. cerevisiae CDC55*, a regulatory subunit of protein phosphatase 2A.

*Corresponding author.
†Present address: Institute für Pflanzenwissenschaften, ETH, Universitätstrasse 2, CH-8092 Zürich, Switzerland.
‡Present address: National Institute for Biotechnology and Genetic Engineering, Jhang Road, PO Box 577, Faisalabad, Pakistan.

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Table 1. Yeast strains used in this study.

Strain	Genotype	Source
RS16 (*S. cerevisiae*)	*MAT***a** *ura3-251,328,372 leu2-3,112*	Gaxiola *et al.* (1992)
RS48 (*S. cerevisiae*)	RS16 *hal3::LEU2*	Ferrando *et al.* (1995)
AHY80 (*S. cerevisiae*)	*MAT***a** *cdc55::LEU2 leu2 his3*	Healy *et al.* (1991)
AHY20(*S. cerevisiae*)	*MAT***a** *ura3*	Healy *et al.* (1991)
NCYC2512 (*C. tropicalis*)	Wild type	R. Ali (this work)

The possible function of acidic domains in protein phosphatase-mediated signal transduction is suggested.

MATERIALS AND METHODS

Yeast strains and growth media

The *S. cerevisiae* and *C. tropicalis* strains used in this study are listed in Table 1. Cells were routinely grown in YPD medium (1% yeast extract, 2% Bacto peptone and 2% glucose). Transformants (Ito *et al.*, 1983) were selected by plating on minimal medium (SD) containing 2% glucose, 0·7% yeast nitrogen base without amino acids (Difco), 50 mM-MES adjusted to pH 6·0 with Tris and supplemented with the indicated requirements (leucine 100 μg/ml, uracil 30 μg/ml or histidine 30 μg/ml). YPD and SD were solidified with 2% agar. Growth on high salt medium was tested on YPD supplemented with 1 M-NaCl by a drop assay (Gaxiola *et al.*, 1992; Ferrando *et al.*, 1995).

Construction and screening of a genomic DNA library from C. tropicalis *in YEP351*

Genomic DNA from *C. tropicalis* strain NCYC2512 was prepared and a library for cloning in *S. cerevisiae* constructed basically as described by Rose (1987). The genomic DNA was partially digested with *Sau*3A and fragments between 7–8 kb in size were selected by agarose electrophoresis, freeze-squeeze purified (Tautz and Renz, 1983) and ligated to *Bam*HI-digested, alkaline phosphatase-treated YEp351, a shuttle plasmid derived from the 2 μ circle and with the *LEU2* gene as marker (Hill *et al.*, 1986). After electroporation-mediated transformation (Dower *et al.*, 1988) of *Escherichia coli* strain WM1100 (a *recA* derivative of MC1061; Miller, 1987), 50 000 ampicillin-resistant colonies were obtained, pooled and stored in 15% glycerol at −70°C. The complete reading frame of *HAL3* was amplified by polymerase chain reaction (PCR) as described (Ferrando *et al.*, 1995), labelled with ^{32}P by the random-priming method (Feinberg and Vogelstein, 1983) and used as a probe to screen the library by colony hybridization (Hanahan and Meselson, 1980). High stringency conditions were employed for hybridization (65°C, 0·8 M-NaCl ionic strength equivalent) and washes (65°C, 80 mM-NaCl ionic strength equivalent). Two colonies, out of 10 000 tested, cross-hybridized with *HAL3*. Plasmid DNA was isolated and cross-hybridizing restriction fragments were subcloned into pBluescript (Stratagene). Unidirectional nested deletions were generated with exonuclease III and S1 nuclease (Henikoff, 1984) according to the 'Erase-a-Base' system of Promega (Madison, Wisconsin). Sequencing was by the dideoxy method and T7 DNA polymerase (Tabor and Richardson, 1987) according to the Sequenase system of USB (Cleveland, Ohio).

Expression of CtCDC55 *in* S. cerevisiae

The *C. tropicalis CDC55* (*CtCDC55*) gene was expressed in *S. cerevisiae* from its own promoter. The 3 kb *Xho*I-*Bgl*II fragment hybridizing with *HAL3* (Figure 1) was subcloned into pBluescript KS (Stratagene, La Jolla, California) digested with *Xho*I and *Bam*HI. *C. tropicalis* DNA was liberated from the resulting plasmid as a *Xho*I-*Sac*I fragment and subcloned into yeast centromeric plasmid pUN90 (*HIS3* marker; Elledge and Davis, 1988) digested with *Sal*I and *Sac*I to produce pUN90-*CtCDC55*. Both pUN90 and pUN90-*CtCDC55* were transformed (Ito *et al.*, 1983) into yeast strain AHY80 (*cdc55 his3*; Healy *et al.*, 1991) to test for complementation of the *cdc55* mutation.

Expression of CtHAL3 *in* S. cerevisiae

The *C. tropicalis HAL3* gene (*CtHAL3*) was expressed in *S. cerevisiae* from its own promoter. The 3 kb *Eco*RI fragment hybridizing with *HAL3* (Figure 1) was subcloned into YEp352, a shuttle

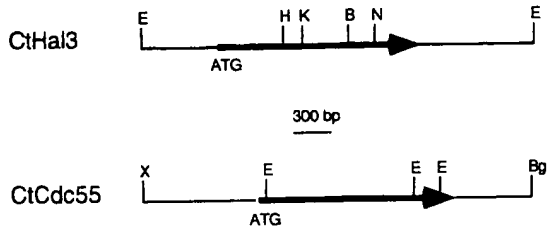

Figure 1. Restriction map of two genomic regions of *C. tropicalis* containing ORFs (arrows) with long acidic domains. *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N) and *Xho*I (X) sites are indicated.

plasmid derived from the 2 μ circle and with *URA3* as marker (Hill *et al.*, 1986), to produce YEp352-*CtHAL3*. Both YEp352 and YEp352-*CtHAL3* were transformed (Ito *et al.*, 1983) into yeast strains RS16 (Hal3$^+$) and RS48 (hal3) to test for salt tolerance. As a comparison, plasmid YEp352-*ScHAL3* (Ferrando *et al.*, 1995) was also transformed into the same strains.

CtHAL3 was also expressed in *S. cerevisiae* from the strong *PMA1* promoter (Serrano and Villalba, 1995). The complete reading frame of *CtHAL3* was PCR-amplified with upstream primer 5'-GGCCGGCTCGAGATGCCTTCTGATAAGG ATATT and downstream primer 5'-GGGC CCCTCGAGTCAAAGGTTAGTAGTTTCATC (both introduce a *Xho*I site, underlined). After digestion with *Xho*I, the 1·6 kb PCR fragment was subcloned with the right orientation into the *Xho*I site of yeast expression plasmid pRS699 (Serrano and Villalba, 1995), to produce pRS699-*CtHAL3*. This plasmid was transformed into yeast strains as described above.

Cloning and expression in S. cerevisiae *of YKL088w*

The open reading frame (ORF) YKL088w of yeast chromosome XI (Dujon *et al.*, 1994) has significant homology to *HAL3* (Ferrando *et al.*, 1995). It was cloned by performing PCR on *S. cerevisiae* genomic DNA (Rose, 1987). The upstream primer was 5'-CCGGCCCTCGAG ATGACGGATGAAAAAGTGAAC and the downstream primer 5'-CGCGCGCTCGAGTT AAACTTCGGTTTTCACGTC (both introduce a *Xho*I site, underlined). Amplification was performed by 30 cycles of incubations at 94°C for 1 min, 50°C for 1 min and 72°C for 3 min. After digestion with *Xho*I, the PCR product (1·7 kb) was subcloned into the *Xho*I site of yeast expression plasmid pRS699 (Serrano and Villalba, 1995). A plasmid (pRS699-YLK088w) was selected with the YKL088w ORF under control of the constitutive *PMA1* promoter and transformed into yeast strains as described above. A positive control plasmid (pRS699-*ScHAL3*) was constructed with the ORF of *S. cerevisiae HAL3* under control of the *PMA1* promoter. The construct was made as described above for YKL088w. The upstream PCR primer as 5'-GGCCGGCTCGAGATGAC TGCCGTCGCCTCTACT and the downstream PCR primer 5'-GGGCCCCTCGAGTTATTGA TGCTTATCTATTAT (both introduce a *Xho*I site, underlined).

RESULTS AND DISCUSSION

Cloning and identification of CtHAL3 *and* CtCDC55

Approximately 10 000 colonies from the *C. tropicalis* genomic library were screened by their ability to cross-hybridize to a *S. cerevisiae HAL3* probe. Two cross-hybridizing clones were identified (Figure 1). One of them contained a 3 kb *Eco*RI cross-hybridizing fragment. Sequencing revealed a novel *C. tropicalis* gene, *CtHAL3*, which encoded a protein of 531 amino acids with significant homologies to Hal3p at its second half (Figure 2). Also included in Figure 2 is the ORF YKL088w (571 amino acids) of *S. cerevisiae* chromosome XI (Dujon *et al.*, 1994), which also has significant homology to *HAL3* at its second half (Ferrando *et al.*, 1995). Altogether these three genes define a novel family of proteins containing a conserved domain of about 200 amino acids at the carboxyl-terminal half. The most conserved motifs within this domain are the polar sequence ELR(R,K)WAD, the cysteine region (G(I,L) C(N,D)NLLT, the glycine-rich loop GDIG (L,K)GG and an acidic tail with 40–50 aspartates and glutamates (Figure 2). There is no significant homology within the amino-terminal half of the proteins.

Plasmid DNA corresponding to the second clone contained a 3 kb *Xho*I-*Bgl*II cross-hybridizing fragment (Figure 1). Sequencing revealed a new *C. tropicalis* gene, *CtCDC55*, which encodes a protein of 509 amino acids highly homologous to the product of the *CDC55* gene of *S. cerevisiae* (Figure 3; Healy *et al.*, 1991). *CtCDC55* contains an internal acidic domain responsible for the cross-hybridization signal to

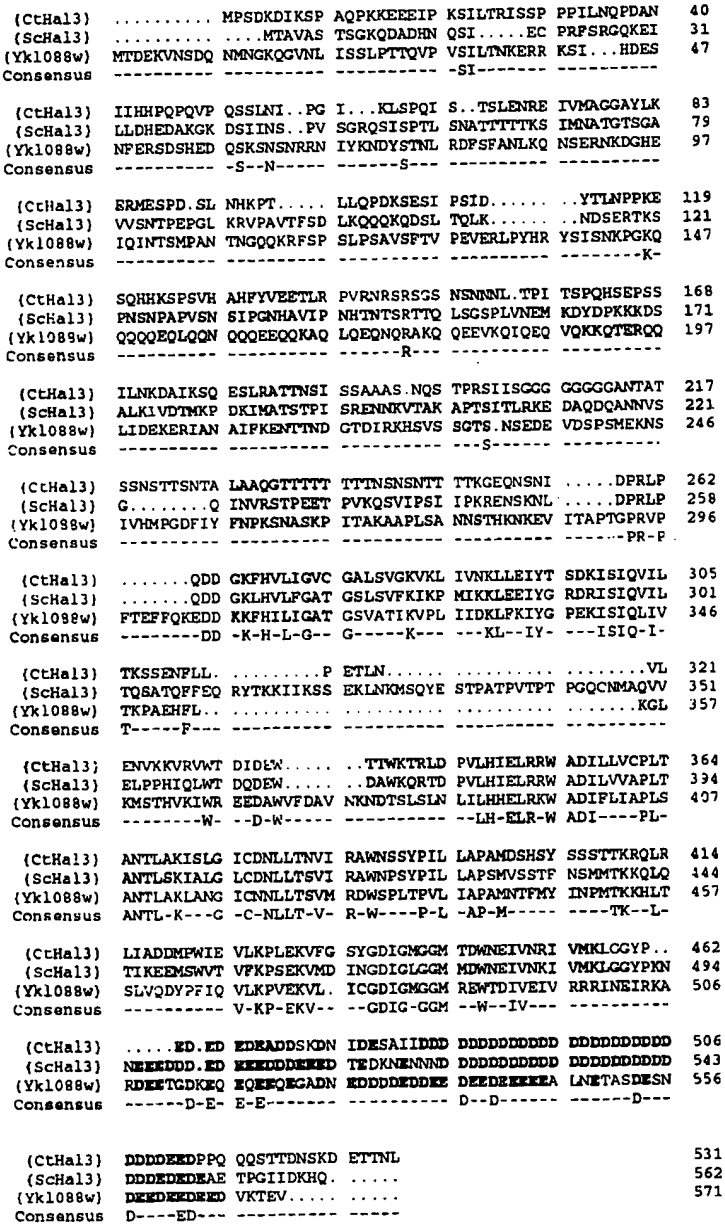

Figure 2. Comparison of the *C. tropicalis HAL3* protein (CtHal3) with the *S. cerevisiae HAL3* protein (ScHal3) and with the ORF YKL088w predicted protein (YKL088w). DNA and deduced protein sequences were analysed using the GCG software package.

HAL3 and which is not present in *CDC55*. Outside this acidic domain, *CtCDC55* has no significant homology to *HAL3*.

S. cerevisiae CDC55 encodes the PR55 subunit (or regulatory subunit B) of protein phosphatase 2A, one of the major serine/threonine-specific phosphatases (Shenolikar, 1994). The core enzyme consists of a 36 kDa catalytic subunit and a 65 kDa regulatory subunit (PR65 or subunit A). It associates with a third, variable regulatory subunit of either 55 (PR55 or subunit B), 72 (PR72 or subunit C) or 130 (PR130 or subunit C') kDa.

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(DmCdc55)  MGRWGRQSPV LEPPDPQMQT TPPPPTLPPR TFMRQSSITK IGNMLNTAIN   50
(HsCdc55)  .......... .......... .......... .......... ..........
(CtCdc55)  .......... .......... .......... .......... ..........
(ScCdc55)  .......... .......... .......... .......... ..........

(DmCdc55)  INGAKKPASN GEASWCFSQI KGALDD..DV TDADIISCVE FNHDGELLAT   98
(HsCdc55)  MAGA...GGG NDIQWCFSQV KGAVDD..DV AEADIISTVE FNHSGELLAT   45
(CtCdc55)  .......... ..MNLDFSQC FGDKGDIENI TEADIISTVE FDHTGDFLAT   38
(ScCdc55)  .......MAQ NNFDFKFSQC FGDKADIV.V TEADLITAVE FDYTGNYLAT   42
Consensus  ---------- ------FSQ- -G---D---- --AD-I--VE F---G--LAT

(DmCdc55)  GDKGGRVVIF QRDPASKAAN PRRGEYNVYS TFQSHEPEFD YLKSLEIEEK  148
(HsCdc55)  GDKGGRVVIF QQEQENKIQS HSRGEYNVYS TFQSHEPEFD YLKSLEIEEK   95
(CtCdc55)  GDKGGRVVLF ERNQSKKKQS ...CEYKFFT EFQSHDAEFD YLKSLEIEEK   85
(ScCdc55)  GDKGGRVVLF ERSNSRH... ...CEYKFLT EFQSHDAEFD YLKSLEIEEK   86
Consensus  GDKGGRVV-F ---------- ----EY---- -FQSH--EFD YLKSLEIEEK

(DmCdc55)  INKIRWLQQK NPVHFLLSTN DKTVKLWKVS ERDKSFGGYN TKEE......  192
(HsCdc55)  INKIRWLPQK NAAQFLLSTN DKTIKLWKIS ERDKRPEGYN LKEE......  139
(CtCdc55)  INKIKWLKSA NDSLCLLSTN DKTIKLWKIQ ERQIKLVSEN NLNGLNHLPS  135
(ScCdc55)  INEIKWLRPT QRSHFLLSTN DKTIKLWKVY EKNIKLVSQN NLTEGVTFAK  136
Consensus  IN-I-WL--- -----LLSTN DKT-KLWK-- E--------N ----------

(DmCdc55)  .......... NGLIRDPQNV TALRVPSVKQ IPLLVEASPR RTFANAHTYH  232
(HsCdc55)  .......... DGRYRDPTTV TTLRVPVFRP MDLMVEASPR RIFANAHTYH  179
(CtCdc55)  SN........ .......IGI ESLKLPQLQL HDKLISAQPK KIYANAHAYH  170
(ScCdc55)  KGKPDNHNSR GGSVRAVLSL QSLKLPQLSQ HDKIIAATPK RIYSNAHTYH  186
Consensus  ---------- ---------- --L--P---- ------A-P- ----NAH-YH

(DmCdc55)  INSISVNSDQ ETFLSADDLR INLWHLEVVN QSYNIVDIKP TNMEELTEVI  282
(HsCdc55)  INSISINSDY ETYLSADDLR INLWHLEITD RSFNIVDIKP ANMEELTEVI  229
(CtCdc55)  INSISVNSDQ ETYLSADDLR INLWNLGIAD QSFNIVDIKP ANMEELTEVI  220
(ScCdc55)  INSISLNSDQ ETFLSADDLR INLWNLDIPD QSFNIVDIKP TNMEELTEVI  236
Consensus  INSIS-NSD- ET-LSADDLR INLW-L---- -S-NIVDIKP -NMEELTEVI

(DmCdc55)  TAAEFHPTEC NVFVYSSSKG TIRLCDMRSA ALCDRHSKQF EEPENPTNRS  332
(HsCdc55)  TAAEFHPNSC NTFVYSSSKG TIRLCDMRAS ALCDRHSKLF EEPEDPSNRS  279
(CtCdc55)  TSAEFHPLQC NLFMYSSSKG TIKLSDMRSN SLCDSHAKIF EEYLDPSSHN  270
(ScCdc55)  TSAEFHPQEC NLFMYSSSKG TIKLCDMRQN SLCDNKTKTF EEYLDPINHN  286
Consensus  T-AEFHP--C N-F-YSSSKG TI-L-DMR-- -LCD---K-F EE---P----

(DmCdc55)  FFSEIISSIS DVKLSNSGRY MISRDYLSIK VWDLHMETKP IETYPVHEYL  382
(HsCdc55)  FFSEIISSIS DVKFSHSGRY MMTRDYLSVK IWDLNMENRP VETYQVHEYL  329
(CtCdc55)  FFTEITSSIS DVKFSHDGRY IASRDYMTVK IWDLAMENKP IKTIDVHEHL  320
(ScCdc55)  FFTEITSSIS DIKFSPNGRY IASRDYLTVK IWDVNMDNKP LKTINIHEQL  336
Consensus  FF-EI-SSIS D-K-S--GRY ---RDY---K -WD--M---P --T---HE-L

(DmCdc55)  RAKLCSLYEN DCIFDKFECC WNGKDSSIMT GSYNNFFRVF ..........  422
(HsCdc55)  RSKLCSLYEN DCIFDKFECC WNGSDSVVMT GSYNNFFRMF ..........  369
(CtCdc55)  RERLCDTYEN DAIFDKFEVQ FGGDNKSVMT GSYNNQFVIY PNAVNTGNDD  370
(ScCdc55)  KERLSDTYEN DAIFDKFEVN FSGDSSSVMT GSYNNNFMIY PNVVTSGDND  386
Consensus  ---L---YEN D-IFDKFE-- --G-----MT GSYNN-F--- ----------

(DmCdc55)  .......... .DRNSKK... .......... .......... ..........  428
(HsCdc55)  .......... .DRNTKR... .......... .......... ..........  375
(CtCdc55)  KPKFKSAFKN SSKRSKKNGF STRTTDDDDD DDDDDDDEEA DDEFDEEVPA  420
(ScCdc55)  NGIVKTFDEH NAPNSNSNKN IHNSIQNKDS SSSGNSHKRR SNGRNTGMVG  436
Consensus  ---------- ---------- ---------- ---------- ----------

(DmCdc55)  .......... .......... .....DVTLE ASRDIIKPK. .TVLKPRKVC  451
(HsCdc55)  .......... .......... .....DITLE ASRENNKPR. .TVLKPRKVC  397
(CtCdc55)  TKNSPGSQLE DDD..... ..EQEEIILQ ADKSAFKSKK SGQHPMRRRM  461
(ScCdc55)  SSNSSRSSIA GGEGANSEDS GTEMNEIVLQ ADKTAFRNKR YGSLAQR...  483
Consensus  ---------- ---------- --------L- A--------- ------R---

(DmCdc55)  TGGKRKKDEI SVDCLDFNKK ILHTAWHPEE NIIAVAATNN LFIFQDKF.   499
(HsCdc55)  ASGKRKKDEI SVDSLDFNKK ILHTAWHPKE NIIAVATTNN LYIFQDKVN  447
(CtCdc55)  TSGVGSNLGR EFDDVDFKKS ILHLSWHPRE NSVAIAATNN LYIFSTL..  508
(ScCdc55)  .SARNKDWG. ..DDIDFKKN NLHFSWHPRE NSIAVAATNN LFIFSAL..  526
Consensus  ---------- --D--DF-K- -LH--WHP-E N--A-A-TNN L-IF-----
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Figure 3. Comparison of the *C. tropicalis CDC55* protein (CtCdc55) with the homolog B subunits of protein phosphatase 2A from *Drosophila melanogaster* (DmCdc55; Mayer-Jaekel *et al.*, 1993), human α isoform (HsCdc55; Mayer *et al.*, 1991) and *S. cerevisiae* (ScCdc55; Healy *et al.*, 1991). The acidic domain of CtCDC55p is shown in bold face. DNA and deduced protein sequences were analysed using the GCG software package.

All subunits are conserved from yeast to man (Mayer *et al.*, 1991; Mayer-Jaeckel *et al.*, 1993; Mayer-Jaekel and Hemmings, 1994). Comparison of some PR55 proteins shows this high conservation (Figure 3). However, a distinctive feature of the product of the *CtCDC55* gene is the presence of an acidic domain. As compared to animal PR55s, both the *C. tropicalis* and *S. cerevisiae* homologs contain an insertion of about 70 amino acids, which in *C. tropicalis*, but not in *S. cerevisiae*, includes 18 aspartates and 8 glutamates (in bold in Figure 3). This acidic domain is 70 amino acids away from the carboxyl terminus.

Southern blot analysis of genomic DNA (results not shown) confirmed the presence of single *CtHAL3* and *CtCDC55* genes in the *C. tropicalis* genome. Accession numbers in EMBL nucleotide sequence data base are X88900 and X88899 for *CtHAL3* and *CtCDC55*, respectively.

CtHAL3p and the predicted S. cerevisiae *YKL088w protein partially complement the salt sensitivity of a* hal3::LEU2 S. cerevisiae *strain*

Disruption of *HAL3* in *S. cerevisiae* results in salt sensitivity (Ferrando *et al.*, 1995). In order to test whether *CtHAL3* and YKL088w (see above) are functional homologs to *HAL3*, we have tried to complement this *hal3* phenotype by expression of the genes (Figure 4). Both *CtHAL3* (panel 3) and *YKL088w* (panel 7) complemented the salt sensitivity of a *hal3* mutant, suggesting that these genes encode proteins with similar activities to *S. cerevisiae* Hal3p. Overexpression of *S. cerevisiae HAL3* with the same plasmids results in higher salt tolerance (panels 4 and 8). However, as the salt tolerance effect of *HAL3* is dose dependent (Ferrando *et al.*, 1995), the relative activities of the different proteins cannot be compared without information on their expression levels. Expression of *CtHAL3* from the strong *PMA1* promoter (pRS699-*CtHAL3*, see Materials and Methods) did not improve the salt tolerance effect of the gene expressed from its own promoter (data not shown).

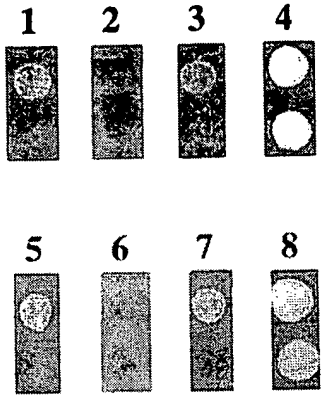

Figure 4. Complementation assay of the *S. cerevisiae hal3* mutant. Drops (3 µl) of 1/10 (top) and 1/100 (bottom) dilutions of saturated cultures were spotted onto YPD plates supplemented with 1 M-NaCl and incubated at 28°C for 5 days. (1) Wild-type strain RS16 (*HAL3*) transformed with control plasmid YEp352; (2–4) strain RS48 (*hal3::LEU2*) transformed with plasmids YEp352 (2), YEp352-*CtHAL3* (3) or YEp352-*ScHAL3* (4); (5) strain RS16 (*HAL3*) transformed with control plasmid pRS699; (6–8) strain RS48 (*hal3::LEU2*) transformed with plasmids pRS699 (6), pRS699-YLK088w (7) or pRS699-*ScHAL3* (8). Identical results were obtained with three different transformants from every plasmid.

CtCDC55 *encodes a functional homolog of* S. cerevisiae CDC55

S. cerevisiae cdc55 mutants display a cold-sensitive phenotype characterized by morphogenetic defects at low temperature (Healy *et al.*, 1991). To test whether *CtCDC55* is a functional homolog of *CDC55*, a complementation assay was carried out. *S. cerevisiae* strain AHY80 (*cdc55::LEU2* disruption mutant) was transformed with a pUN90 centromeric vector containing the 3 kb *XhoI/BglII CtCDC55* fragment and growth was tested at 28°C and 14°C (Figure 5A). The *cdc55* mutant (columns 1 and 3) shows a growth delay at low temperature as compared to wild type (column 2). However, after being transformed with the *CtCDC55* gene, it was able to grow at 14°C as wild type. The aberrant morphology developed at low temperature by the *cdc55* mutant (Figure 5B, panel 3) was also reverted by the *CtCDC55* gene (Figure 5B, panel 4). This demonstrates that *CtCDC55* is a true functional homolog of *S. cerevisiae CDC55*.

Long acidic domains in putative regulatory proteins

S. cerevisiae Hal3p (Ferrando *et al.*, 1995), CtHal3p and CtCdc55p (present work) are examples of putative regulatory proteins with long acidic domains. Domains with more than 20 glutamates and/or aspartates have been identified in nuclear proteins such as centromere protein CENP-B, non-histone proteins HMG-1,2, nucleolin and nucloplasmin (Earnshaw, 1987). These domains are much more acidic than activator

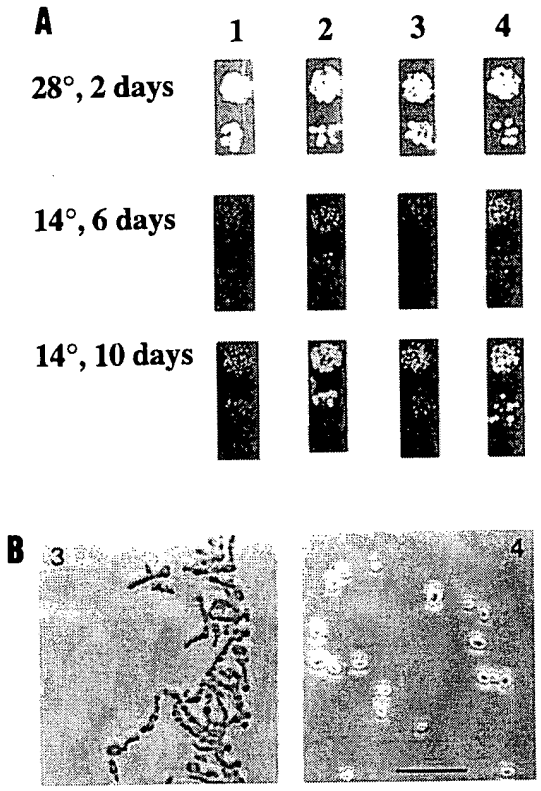

Figure 5. Complementation assay of the *S. cerevisiae cdc55* mutant. (A) Drops (3 µl) of 1/10 (top) and 1/100 (bottom) dilutions of saturated cultures were spotted onto YPD plates and incubated at 28°C or 14°C for the days indicated. (1) Strain AHY80 (*cdc55:LEU2*); (2) wild-type strain AHY20 (*CDC55*); (3) strain AHY80 transformed with control plasmid pUN90; (4) strain AHY80 transformed with pUN90-*CtCDC55*. Identical results were obtained with three different transformants from every plasmid. (B) Morphology of strain AHY80 (*cdc55::LEU2*) transformed with pUN90 or pUN90-*CtCDC55*. Phase contrast photomicrographs (Nikon104 microscope) were taken of the colonies shown in panel 1, lanes 3 and 4, after 10 days at 14°C. Magnification bar corresponds to 35 µm.

domains of transcription factors such as Gal4p and Gcn4p. The UBF transcription factor has a long acidic tail which may participate in nucleolar targeting (Maeda *et al.*, 1992) and a general role for acidic domains in unfolding chromatin structure by electrostatic 'capture' of histones has been proposed (Earnshaw, 1987). Calsequestrin and calreticulin, calcium-binding proteins of animal (Fliegel *et al.*, 1987; Michalak *et al.*, 1992) and plant (Krause *et al.*, 1989; Menegazzi *et al.*, 1993) microsomes contain a long acidic tail involved in low-affinity calcium binding and in retention within the lumen of the endoplasmic reticulum (Michalak *et al.*, 1992). Therefore, acidic domains of nuclear and microsomal proteins may have multiple functions.

The acidic tail of *S. cerevisiae* Hal3p is essential for its salt tolerance activity (Ferrando *et al.*, 1995). Hal3p has no signal peptide and is not a microsomal protein but is probably located at the cytoplasm (Ferrando *et al.*, 1995) and/or nucleus (Di Como *et al.*, 1995). Calcium binding to Hal3p measured by a $^{45}Ca^{2+}$ overlay assay (Krause *et al.*, 1989) gave negative results (A. Ferrando and R. Serrano, unpublished observations) and genetic evidence indicates that Hal3p does not participate in the transduction of the salt stress signal mediated by calcineurin and which probably involves calcium changes (Marquez and Serrano, 1996). Therefore, a role of the acidic domain of Hal3p in protein–protein interactions seems more likely than calcium binding. In this respect it can be mentioned that the cytoplasmic Hsp90 chaperone contains an acidic region that is thought to interact with several steroid hormone receptors (Binart *et al.*, 1995).

Overexpression of *S. cerevisiae HAL3/SIS2* suppresses both the salt sensitivity conferred by lack of the protein phosphatase 2B calcineurin (Ferrando *et al.*, 1995) and the reduced expression of G1 cyclins conferred by lack of the protein phosphatase 2A Sit4p (Di Como *et al.*, 1995). Therefore, it has been proposed that Hal3p is a regulatory subunit of some unidentified protein phosphatase (Ferrando *et al.*, 1995). Its essential acidic tail could mediate binding to the catalytic subunit of the phosphatase. In this respect, CtCdc55p is the first example of a phosphatase regulatory subunit containing a long acidic domain. A plausible function for acidic domains in regulatory proteins is that they constitute one type of module for interactions between subunits of protein complexes. It could be predicted that an acidic module in one protein would have a matching basic module in the interacting protein. In this respect, the acidic N-terminus of immunophilin FKBP46 has been described to interact with basic nuclear protein TP2 (Alnemri *et al.*, 1994). The identification of the catalytic subunit of protein phosphatase 2A in *C. tropicalis* and of the proteins interacting with Hal3p could provide additional evidence for this hypothesis. It must be indicated, however, that Cdc55p from *S. cerevisiae* does not contain an acidic domain (Healy *et al.*, 1991) and therefore electrostatic interactions between

domains are not essential for regulation of protein phosphatase 2A.

In addition to protein phosphatase complexes, acidic domains could mediate interactions within other types of regulatory complexes such as those nucleated by protein kinases. A subfamily of plant protein kinases has been described which contain acidic tails and which are induced by osmotic and temperature stresses (Holappa and Walker-Simmons, 1995). It would be interesting to investigate the role of this acidic domain in mediating interactions of the catalytic subunit of protein kinases with other regulatory subunits.

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Annex 1: Multiple alignment of VB89 (SEQ ID NO: 8) with yeast HAL3 (HAL3p_Sc), ScKLY088w and Candida HAL3 (CtHAL3). Conserved residues are in bold.

```
   MSF:  654  Type: P    Check:  3002   ..
Name: VB89 oo        Len:  654  Check:  6450  Weight:  1.000
Name: HAL3p_Sc oo    Len:  654  Check:  4750  Weight:  1.000
Name: CtHAL3 oo      Len:  654  Check:  2554  Weight:  1.000
Name: SCYKL088W oo   Len:  654  Check:  9248  Weight:  1.000

//
VB89            .......... .......... .......... .......... ..........
HAL3p_Sc        ....MTAVAS TSGKQDA... ........DH NQSI.....E CPRFSRGQ..
CtHAL3          MPSDKDIKSP AQPKKEE... ........EI PKSILTRISS PPPILNQP..
SCYKL088W       .MTDEKVNSD QNMNGKQGVN LISSLPTTQV PVSILTNKER RKSIHDESNF


VB89            .......... .......... .......... .......... ..........
HAL3p_Sc        KEILLDHEDA KGKDSIINSP VSGRQSISPT LSNA..TTTT TKSIMNATGT
CtHAL3          DANIIHHPQP QVPQSSLNIP ...GIKLSPQ IS....TSLE NREIVMAGGA
SCYKL088W       ERSDSHEDQS KSNSNRRNIY ...KNDYSTN LRDFSFANLK QNSERNKDGH


VB89            .......... .......... .......... .......... ..........
HAL3p_Sc        SGAVVSNTPE PGLKRVPAVT FSDLKQQQ.. ........KQ DSLTQLKNDS
CtHAL3          YLKERMESPD .SLNHKPT.. ...LLQPD.. ........KS ESIPSIDYTL
SCYKL088W       EIQINTSMPA .NTNGQQK.. ...RFSPSLP SAVSFTVPEV ERLPYHRYSI


VB89            .......... .......... .......... .......... ..........
HAL3p_Sc        ERTKSPNSNP APVSNSIPGN HAVIPNH... .TNTSRTTQL SGSPLVNEMK
CtHAL3          NPPKESQHHK SPSVHA.... HFYVEET... .LRPVRNRSR SGSNSNNNLT
SCYKL088W       SNKPGKQQQQ QEQLQQ.... NQQQEEQQKA QLQEQNQRAK QQEEVKQIQE


VB89            .......... .......... .......... .......... ..........
HAL3p_Sc        DYDPKKKDSA LKIV..DTMK PDKIMATSTP ISRENNKVTA KAPTSITLRK
CtHAL3          PITSPQHSEP SSILNKDAIK SQESLRATTN SISSAAASNQ STPRSIISGG
SCYKL088W       QVQKKQTERQ QLIDEKERIA NAIFKENTTN DGTDIRKHSV SS........


VB89            .......... .......... .......... .......... ..........
HAL3p_Sc        EDAQDQANNV SGQ....... .......... .......... ...INVRSTP
CtHAL3          GGGGGGANTA TS........ .......... ....SNSTTS NTALAAQGTT
SCYKL088W       ..GTSNSEDE VDSPSMEKNS IVHMPGDFIY FNPKSNASKP ITAKAAPLSA


VB89            .....MNMEV DTVTR.KP.. .......... ....RILLAA SGSVASIKFS
HAL3p_Sc        EETPVKQSVI PSIIPKRENS KNLDPRLPQD DGKLHVLFGA TGSLSVFKIK
CtHAL3          TTTTTTNSNS NTTTTKGEQN SNIDPRLPQD DGKFHVLIGV CGALSVGKVK
SCYKL088W       NNSTHKNKEV ITAPT.GPRV PFTEFFQKED DKKFHILIGA TGSVATIKVP


VB89            NLCHCF.... .SEWAEVKAV ASKSSLNFVD K......... .PSLP.....
HAL3p_Sc        PMIKKLEEIY GRDRISIQVI LTQSATQFFE QRYTKKIIKS SEKLNKMSQY
CtHAL3          LIVNKLLEIY TSDKISIQVI LTKSSENFLL P......... .ETLN.....
SCYKL088W       LIIDKLFKIY GPEKISIQLI VTKPAEHFLK G......... .LKMS.....


VB89            .......... .......... ....QNVTLY TDEDEW.... ...SSWNKIG
HAL3p_Sc        ESTPATPVTP TPGQCNMAQV VELPPHIQLW TDQDEW.... ...DAWKQRT
CtHAL3          .......... .........V LENVKKVRVW TDIDEW.... ...TTWKTRL
```

```
SCYKL088W       .......... .......... ....THVKIW REEDAWVFDA VNKNDTSLSL

VB89            DPVLHIELRR WADVMIIAPL SANTLAKIAG GLCDNLLTCI VRAWDYSKPL
HAL3p_Sc        DPVLHIELRR WADILVVAPL TANTLSKIAL GLCDNLLTSV IRAWNPSYPI
CtHAL3          DPVLHIELRR WADILLVCPL TANTLAKISL GICDNLLTNV IRAWNSSYPI
SCYKL088W       NLILHHELRK WADIFLIAPL SANTLAKLAN GICNNLLTSV MRDWSPLTPV

VB89            FVAPAMNTLM WNNPFTERHL VLL..DELGI TLIPPIKKKL .ACGDYGNGA
HAL3p_Sc        LLAPSMVSST FNSMMTKKQL QTIKEEMSWV TVFKPSEKVM DINGDIGLGG
CtHAL3          LLAPAMDSHS YSSSTTKRQL RLIADDMPWI EVLKPLEKVF GSYGDIGMGG
SCYKL088W       LIAPAMNTFM YINPMTKKHL TSLVQDYPFI QVLKPVEKVL .ICGDIGMGG

VB89            MAEPSLIYST VRLFWESQAR KQRDGTS... .......... ..........
HAL3p_Sc        MMDWNEIVNK IVMK.LGGYP KNNEEEDDDE DEEEDDDEEE DTEDKNENNN
CtHAL3          MTDWNEIVNR IVMK.LGGYP .......... .....EDEDE DEADDSKDNI
SCYKL088W       MREWTDIVEI VRRR.INEIR KARDEETGDK EQEQEEQEGA DNEDDDDEDD

VB89            .......... .......... .......... .......... ..........
HAL3p_Sc        DD....DDDD DDDDDDDDDD DDDDDDDDED EDEAE....T PGIIDKHQ..
CtHAL3          DESAIIDDDD DDDDDDDDDD DDDDDDDDDD DDDEEDPPQQ QSTTDNSKDE
SCYKL088W       EE....DEED EEEEEALNET ASDESNDEED EEDEED.VKT EV........

VB89            ....
HAL3p_Sc        ....
CtHAL3          TTNL
SCYKL088W       ....
```

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Arabidopsis thaliana AtHAL3: a flavoprotein related to salt and osmotic tolerance and plant growth

Ana Espinosa-Ruiz, Jose M. Bellés, Ramón Serrano and Francisco A. Culiáñez-Macià*
Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera s/n, 46022 Valencia, Spain

Summary

We have isolated two *Arabidopsis thaliana* genes, *AtHAL3a* and *AtHAL3b*, showing homology with HAL3, a yeast protein which regulates the cell cycle and tolerance to salt stress through inhibition of the PPZ1 type-1 protein phosphatase. Expression of *AtHAL3a* in yeast *hal3* mutants partially complements their LiCl sensitivity, suggesting possible conserved functions between both proteins. *AtHAL3a* and *AtHAL3b* are induced by salt stress and *AtHAL3a* is the most expressed in non-stressed plants, particularly in seeds. *In situ* hybridization demonstrates enrichment of *AtHAL3a* mRNA in seed embryos and in the vascular phloem of different plant tissues. AtHAL3 proteins show striking homology with a group of proteins found in fungi, plants and animals and some homology with a large family of prokaryotic flavoproteins. Recombinant AtHAL3a protein purified from *Escherichia coli* was yellow because it contained a non-covalently bound chromophore revealed as flavin mononucleotide. Transgenic *Arabidopsis* plants, with gain of *AtHAL3a* function, show altered growth rates and improved tolerance to salt and osmotic stress.

Introduction

Abiotic stresses, particularly drought and salinity, account for major losses in the yield of crop plants (Boyer, 1982). Improving plant traits that allow better adaptation to adverse environmental conditions is a challenge for modern plant biotechnology. Plant molecular biologists and breeders should co-operate in working towards preventing desert land expansion and world starvation.

One approach to drought and salt stress is to determine pathways, genes and metabolites involved in the complex plant response to these stresses (Bray, 1997; Ingram and Bartels, 1996). Another strategy is the isolation of plant genes homologous to those that play a fundamental role in improving drought and salt tolerance in lower organisms (Serrano, 1994; Holmberg and Bülow, 1998).

We were interested in the isolation and characterization of an *Arabidopsis thaliana* gene homologous to the yeast *Saccharomyces cerevisiae SIS2/HAL3* gene (referred to as *HAL3*) (Di Como *et al.*, 1995; Ferrando *et al.*, 1995). Overexpression of *HAL3* improves growth of wild-type cells exposed to toxic concentrations of sodium and lithium. Although the sequence of HAL3 gives little clue about its function, alterations in intracellular cation concentrations associated with changes in *HAL3* expression indicate that HAL3 activity directly increases cytoplasmic K^+ concentration and decreases Na^+ and Li^+ concentrations (Ferrando *et al.*, 1995). In addition, *HAL3* overexpression partially relieves loss of transcription of G1 cyclins in mutants lacking the protein phosphatase Sit4p, a protein required for passage from G1 to S phase in the cell cycle (Di Como *et al.*, 1995).

In this work we describe the isolation and posterior characterization of two *A. thaliana* genes coding for two proteins named AtHAL3a and AtHAL3b. The *Arabidopsis* proteins showed striking homology with yeast HAL3 and with a number of proteins from different organisms. Overexpression of *AtHAL3a* in the yeast *hal3* mutant partially complemented the salt sensitivity of the mutant. Recombinant AtHAL3a purified from *E. coli* was yellow and shown to be a flavin mononucleotide (FMN) flavoprotein. Finally, transgenic *Arabidopsis* plants overexpressing *AtHAL3a* showed altered growth phenotypes and improved salt and osmotic tolerance.

Results

Molecular cloning of AtHAL3a *and* AtHAL3b

An *Arabidopsis* cDNA (Stock 164P17T7) (R30079) identified in the course of an EXPRESSED SEQUENCE TAGS (ESTs) program (Newman *et al.*, 1994), has a deduced amino-acid sequence 52% identical to the yeast HAL3 protein. By using this cDNA as a probe, two genes with homology to yeast *HAL3* were isolated from an *A. thaliana* genomic library and named *AtHAL3a* and *AtHAL3b* (Figure 1a). The first gene corresponded to the cDNA 164P17T7. *AtHAL3a* and *AtHAL3b* genes contained, at the same position, an intron of 115 and 80 bp, respectively, and their open reading frames are 83% identical. Southern analysis of both genes (Figure 1b) demonstrates the expected cross-hybridization and also indicates that *Arabidopsis* does not contain other closely related genes. Despite the similarities within the

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*For correspondence (fax +34 6 3877859; e-mail faculia@ibmcp.upv.es).

open reading frames the similarity between the 5′ flanking regions was very low (41% identity), pointing to differences in expression of both genes.

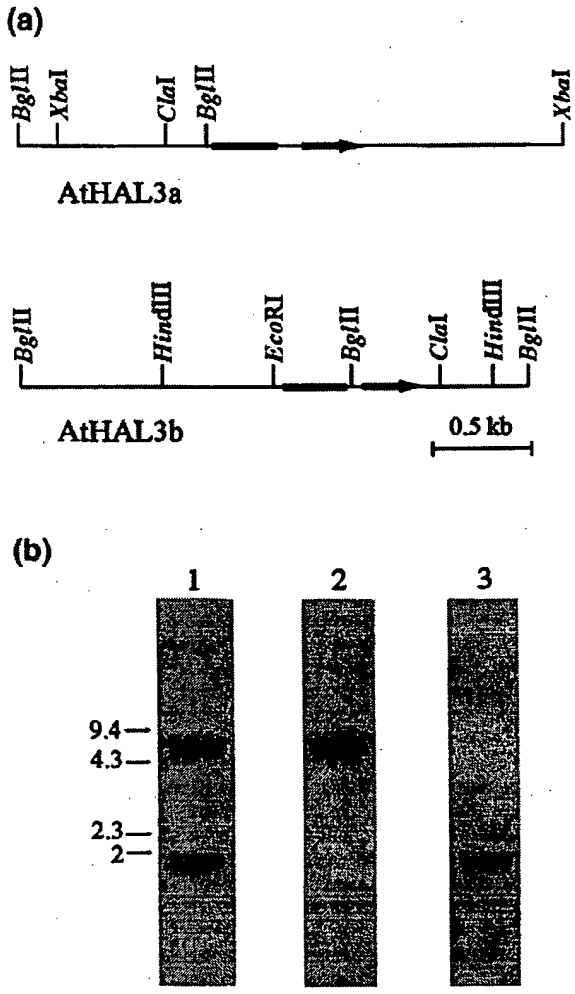


Figure 1. *AtHAL3* gene copy number.
(a) Restriction map of *AtHAL3a* and *AtHAL3b* genomic clones. Translated sequences are shown by thick arrows.
(b) Southern blot of *Arabidopsis* genomic DNA, digested with *Hin*dIII and hybridized with *AtHAL3a* cDNA (1), *AtHAL3a* 3′-untranslated (2) and *AtHAL3b* 3′-untranslated (3) radiolabelled probes at high stringency. Molecular markers are shown on the left in kDa.

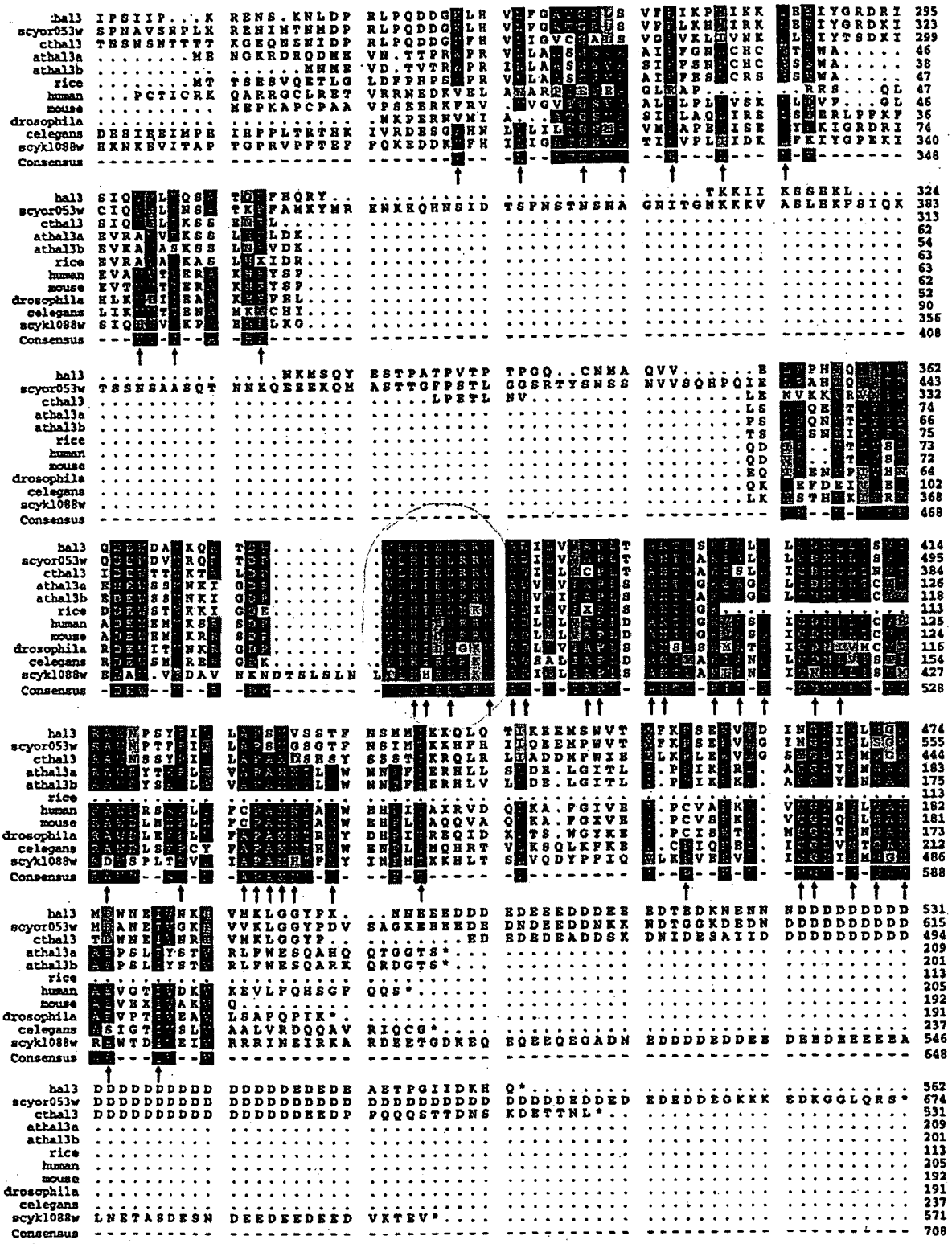
Identification of a novel homologous family

Comparison of the deduced *AtHAL3a* and *AtHAL3b* protein sequences with the Genebank database revealed a striking homology with other eukaryotic sequenced proteins from rice, humans, mouse, *Drosophila melanogaster*, *Caenorhabditis elegans*, *S. cerevisiae* and *Candida tropicalis*. The regions of greatest similarity observed between the plant proteins are also conserved in the consensus derived from all family members (Figure 2). This homology occurs particularly in a central domain expanded along 180 amino-acid residues. Surprisingly, an aspartate- and glutamate-rich domain present in the carboxyl terminus of *C. tropicalis* and *S. cerevisiae* proteins is missing in the other family members. The rice C51401 protein is the most similar to AtHAL3a and AtHAL3b proteins (65–66% amino-acid identity). The plant proteins are more closely related to the animal than to the yeast homologous proteins. The eukaryotic family showed some homology with a large family of prokaryotic flavoproteins including, as key member, the *E. coli* DNA flavoprotein (DFP), involved in DNA replication (Spitzer and Weiss, 1985) and pantothenate metabolism (Spitzer *et al.*, 1988). The amino acids conserved between both families are indicated by arrows in Figure 2. Overall homology between the members of the eukaryotic family, however, is at least 33%, while the homology between eukaryotic and prokaryotic proteins of these families is less than 20%.

Expression of AtHAL3a *and* AtHAL3b

Figure 3 illustrates the expression of the two *Arabidopsis* genes in different tissues and under stress conditions. *AtHAL3a* and *AtHAL3b* expression was examined in roots, shoots, leaves, flowers, developing siliques and seeds (Figure 3a). Transcripts of *AtHAL3a* were detectable on Northern blots of poly(A)+ RNA from all these organs, particularly in flowers, siliques and seeds. The highest expression observed was in dry seeds, pointing to accumulation of *AtHAL3a* mRNA during *Arabidopsis* embryogenesis. The expression of *AtHAL3b* follows the pattern of *AtHAL3a* except in seeds, but the levels of transcript were considerably lower (particularly in seeds).

Figure 2. AtHAL3a and AtHAL3b alignment with homologous proteins.
Alignment of predicted amino-acid sequences for *Arabidopsis* AtHAL3a and AtHAL3b (accession numbers AF166262 and AF166263, respectively) to other related proteins from rice C51401 (C27242), human clone 730510 5′ (AA412663), mouse clone 1382198 5′ (AA798287), *Drosophila* CKO1102 3′ (AA141034), *Caenorhabditis elegans* cosmid (Z81069), yeast SIS2/HAL3 haloprotein (P36024) (Di Como *et al.*, 1995; Ferrando *et al.*, 1995), yeast open reading frame YOR053w (Z74961), *Candida tropicalis* CtHAL3 (X88900) (Rodríguez *et al.*, 1996), and yeast open reading frame YKL088w (Z28088), using the progressive alignment method of Feng *et al.* (1987). Residues are in dark boxes if six of 10 residues at a position are identical. Conserved residues are in grey boxes. The consensus sequence from all family members is shown below. Complete sequences show an asterisk at the end. The consensus homology with a large family of panthotenate metabolism flavoproteins (DFP), including *Escherichia coli* DFP (P24285) (Spitzer and Weiss, 1985), *Haemophilus influenzae* (P44953), *Synechocystis* sp. (D90910), *Bacillus subtilis* (Y13937), *Helicobacter pylori* (AE000595) and *Bradyrhizobium japonicum* (AF042096), is shown by arrows.


hal3
scyor053w
cthal3
athal3a
athal3b
rice
human
mouse
drosophila
celegans
scykl088w
Consensus

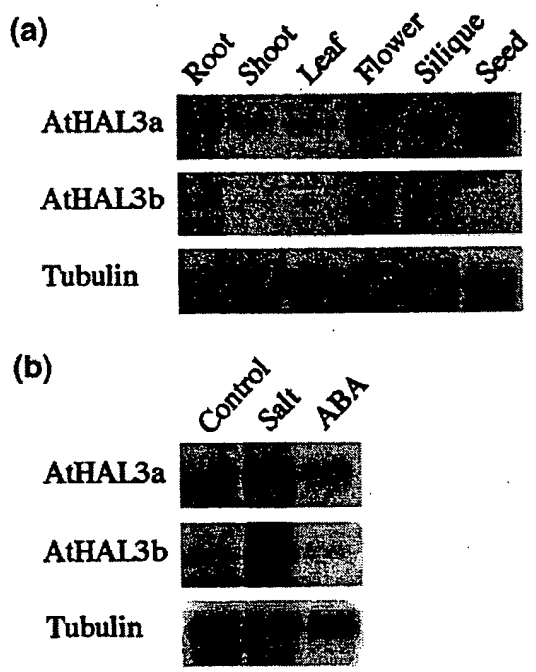


Figure 3. Expression of *AtHAL3a* and *AtHAL3b* mRNA during development and under stress treatments.
Northern blot of *Arabidopsis* poly(A)+ RNA probed with radiolabelled *AtHAL3a* and *AtHAL3b* cDNA.
(a) Different tissues.
(b) 12-day-old seedlings cultivated on MSS medium (control), MSS+100 mM NaCl (salt) and MSS+10 μM ABA.
The same filter was hybridized successively with *AtHAL3b* 3'-untranslated, *AtHAL3a* 3'-untranslated, and tubulin ^{32}P-labelled probes. Tubulin was used as loading control.

AtHAL3a and *AtHAL3b* expression was also examined in seedlings subjected to salt stress (100 mM NaCl) and ABA (10 μM) treatments (Figure 3b). ABA treatment did not show any effect on expression, while salt stress induced the expression of both genes. NaCl was a potent inducer of *AtHAL3b* expression, which under salt stress reached expression levels similar to those of *AtHAL3a*.

Spatial pattern of AtHAL3a *mRNA accumulation during* Arabidopsis *embryogenesis and in vegetative tissues*

Figure 4 shows the spatial and temporal pattern of *AtHAL3a* expression during embryo maturation and in vegetative tissues, further investigated by *in situ* hybridization of *AtHAL3a* mRNA. Fixed paraffin sections were hybridized with digoxigenin-labelled *AtHAL3a* antisense or sense strand probes to locate *AtHAL3a* mRNA sequences. During embryogenesis, *AtHAL3a* mRNA is detected in the cotyledons and hypocotyl of mature seeds; the *AtHAL3a* antisense probe produced intense hybridization, staining being mainly associated with embryo-specialized cell types from hypocotyl and cotyledon provascular tissues; a lower level of hybridization also occurs in the seed coat outer tegument and in the silique epidermis (Figure 4a,b). Accumulation of *AtHAL3a* mRNA was also observed in flower, shoot, leaf and root (Figure 4d–e, g–h, i–k, n–o, respectively), staining being restricted to differentiated cells from vascular elements of the vegetative tissues. In general, it seems that *AtHAL3a* expression is highly enriched in the phloem part of vascular tissue. *In situ* hybridization with a *AtHAL3a* mRNA control probe was used to monitor background hybridization. The specificity of the reaction was shown by the lack of appreciable reaction of the *AtHAL3a* sense-strand probe with the paraffin-embedded sections (Figure 4c,f,i,m,p).

Expression of AtHAL3a *in yeast* hal3 *mutants increases the tolerance to* Li^+

In order to test if *AtHAL3* is a functional homolog of yeast *HAL3*, we have expressed *AtHAL3a* in a yeast strain devoid of functional *HAL3*. As *hal3* mutants are sensitive to sodium and lithium (Ferrando *et al.*, 1995), we have tested if expression of *AtHAL3* complements these phenotypes. One striking difference between yeast HAL3 and *Arabidopsis* AtHAL3 is the presence in the fungal protein of a long acidic tail which has been reported to be essential to improve NaCl tolerance (Ferrando *et al.*, 1995) and to improve the growth of *sit4* mutants (Di Como *et al.*, 1995). Accordingly, we have included in our complementation studies both a truncated yeast HAL3, devoid of the acidic tail, and a chimeric AtHAL3 where the yeast acidic tail was fused to the AtHAL3 coding sequence. Figure 5 shows the lithium tolerance of yeast strain RS48 (*hal3* null mutant) transformed with different constructions including: yeast *HAL3*, yeast *HAL3* without the acidic tail, *AtHAL3a*, and a chimeric gene consisting of *AtHAL3a* fused with the yeast *HAL3* acidic tail. All the constructions were based in the high-copy number vector pRS699, which carries the constitutive strong PMA1 promoter. Cells transformed with empty vector were used as control. The effect of genetic dosage was studied using strain RS16 (wild type) which presents one copy of the wild-type *HAL3* gene. To confirm that all the different transformants grew equally well in the absence of salt stress, growth control tests were performed on YPD solid media (Figure 5a). In 100 mM LiCl (Figure 5b), AtHAL3a partially rescues the salt sensitivity exhibited by the *hal3* mutant; lithium tolerance slightly increases in cells expressing the chimera of *AtHAL3* fused to the yeast *HAL3* acidic tail. Complementation of lithium tolerance with a plasmid containing yeast *HAL3* showed little dependence on the presence of an acidic tail. Essentially identical results were obtained in drop tests with different LiCl concentrations (80, 150 and 200 mM LiCl; data not shown). Tolerance to 1 M NaCl, however, was more demanding on the type of HAL3 construct. AtHAL3a failed to complement the salt sensitivity of the *hal3*

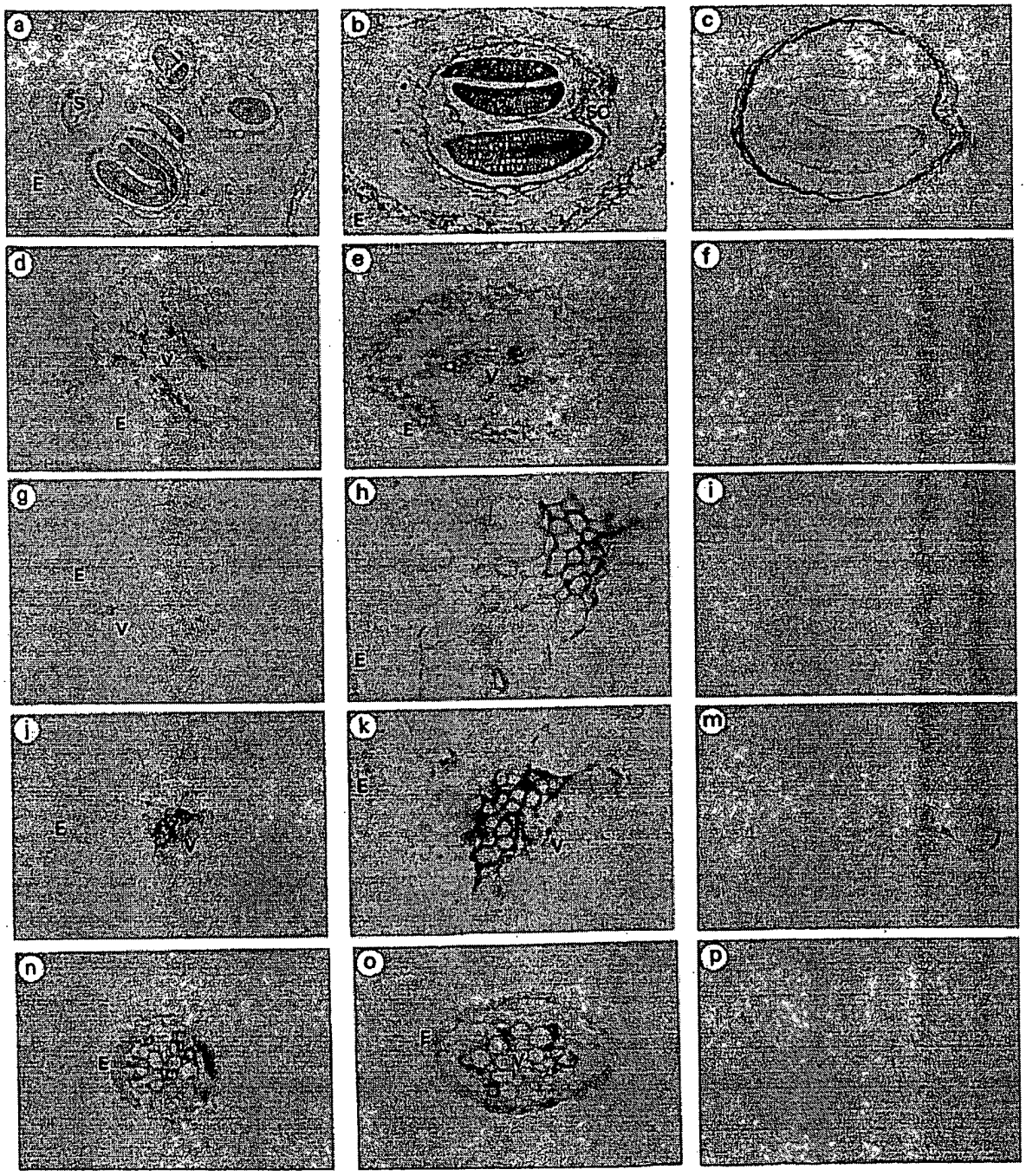


Figure 4. Localization of *AtHAL3* transcripts in seeds and different organs of *Arabidopsis*.
Paraffin-embedded sections hybridized with digoxygenin-labelled antisense *AtHAL3a* and viewed under bright field which gives a blue label.
(a,b) Transversal sections of mature silique; (d,e) longitudinal sections of flower; (g,h) transverse sections of shoot; (j,k) transverse sections of leaf; (n,o) transverse sections of root; (c,f,i,m,p) controls hybridized with *AtHAL3a* sense probe. Abbreviations: E, epidermis; S, seed; SC, seed coat; V, vascular tissue. Magnification ×50 in (a,d,g,j,n); ×100 in (b,c,e,f,h,i,k,m,o,p).

mutant, and the chimera with addition of the yeast acidic tail was also without effect. Essentially identical results were obtained in different NaCl concentrations (0.8 and 1.2 M NaCl, data not shown).

Identification of AtHAL3 as a flavoprotein

In order to characterize AtHAL3 protein, we expressed it in *E. coli* with a poly-histidine tail and purified it by nickel

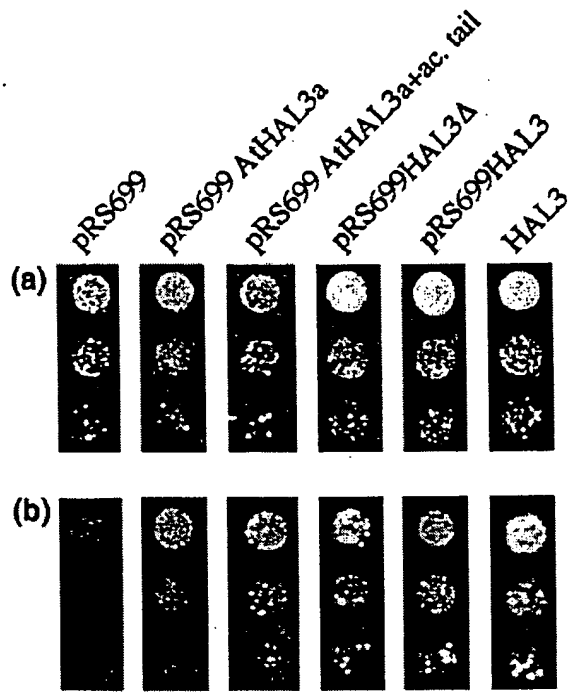


Figure 5. Complementation of the *S. cerevisiae hal3* null mutant with *AtHAL3a*.
(a) YPD solid media; (b) YPD+100 mM LiCl. Drops represent three different dilutions of saturated cultures, 1/10 (top), 1/100 (middle) and 1/1000 (bottom), incubated at 28°C for 36 h (a) and 7 days (b). *hal3* null mutant strain RS48 (RS16 *hal3::LEU2*) was transformed with the high-copy number vector pRS699 carrying different constructions: (pRS699) empty vector; (pRS699 AtHAL3a) *AtHAL3a*; (pRS699 AtHAL3a+ac.tail) *AtHAL3a* fused to *HAL3* acidic tail; (pRS699 HAL3Δ) truncated *HAL3* without acidic tail; (pRS699 HAL3) *HAL3*. (HAL3) wild-type strain RS16. Results were identical using three different transformants of each construct.

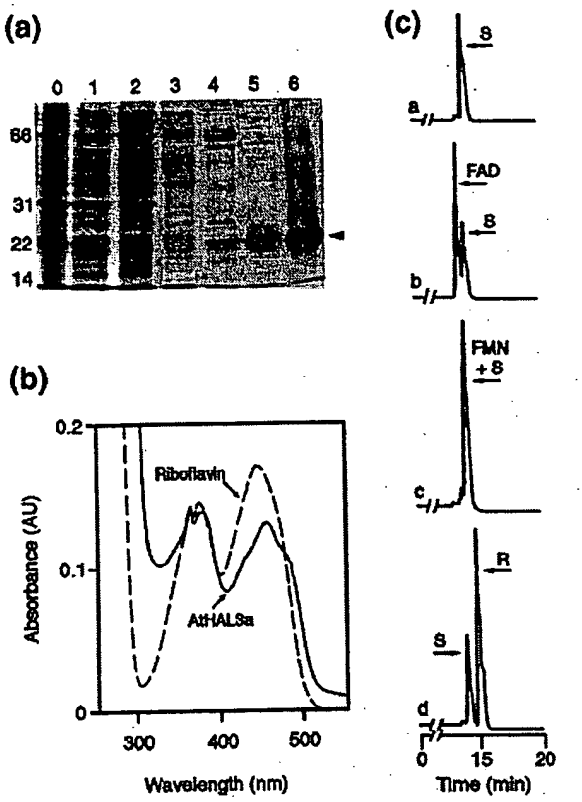


Figure 6. SDS-PAGE analysis of the *AtHAL3a* gene product and spectroscopic characterization of purified AtHAL3a.
(a) SDS-gel electrophoresis of His.Bind metal chelation resin (pET System, Novagen) fractions. The numbers above the tracks refer to the column fractions: (0) total protein extract from *E. coli* transformed with plasmid pET28-AtHAL3 (containing the coding region of the *AtHAL3a* cDNA), before column purification; (1–6) different column-eluted fractions after column purification. The 12% polyacrylamide gel was stained with Coomassie blue. Protein markers are shown on the left in kDa. AtHAL3a is indicated with an arrow.
(b) Absorbance spectrum of the 23 kDa AtHAL3a flavoprotein. The solid line is the spectrum of column fraction 6 from (a). The dashed line is the spectrum of 4 mM riboflavin. Samples were scanned in a Pharmacia 2000 spectrophotometer.
(c) Identification by HPLC of the fluorescent chromophore. 10 µl fraction 6 supernatant after acid treatment was either injected alone (a) or co-injected with 1 nmol of standard FAD (b), FMN (c) or riboflavin (d), respectively. Note the overlap between sample and standard FMN peaks in (c). Abbreviations: S, sample; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; R, riboflavin.

affinity chromatography. Figure 6 shows the SDS-gel electrophoresis of different protein fractions, the absorption spectra of the protein and the coenzyme identification. AtHAL3 was isolated from fraction 6, containing a partially purified protein, that migrated according to the predicted molecular mass (23 kDa) (Figure 6a). This band was absent in the control bacterial strain (data not shown). The most striking feature of the AtHAL3 preparation was its yellow color, pointing to the presence of a chromophore. Partially purified AtHAL3a showed the typical absorption spectrum of an oxidized flavoprotein (Williams, 1976; Kupke *et al.*, 1992), similar to the one showed by a characteristic flavin (riboflavin) (Spitzer and Weiss, 1985) (Figure 6b). The coenzyme released from the protein by heat precipitation, a treatment that should release noncovalently bound fluorescent chromophores (Koziol, 1971; Spitzer and Weiss, 1985), was identified by HPLC as FMN, as assessed by its co-elution with standard FMN (Figure 6c). The same results were obtained under different chromatographic conditions (data not shown). From the specific fluorescence of the preparation, the molar relationship of FMN and protein was 0.8, suggesting one molecule of FMN bound per molecule of AtHAL3a.

Sequence alignments for the cofactor-binding region of the flavin domain of key members of the FMN-containing family, bacterial flavodoxin (Watenpaugh *et al.*, 1973), yeast old yellow enzyme 12-oxophytodienoate reductase (OPDA reductase) (Saito *et al.*, 1991), bacterial oxidoreductase epidermin (EPID) (Kupke *et al.*, 1992), rat liver

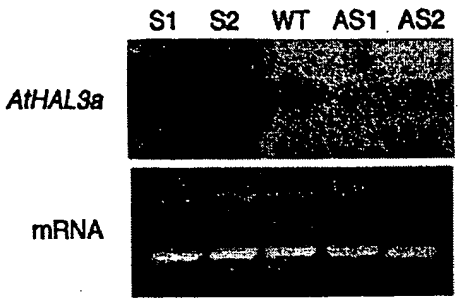


Figure 7. *AtHAL3a* constitutive expression on *Arabidopsis* transgenic lines.
Northern blot of *Arabidopsis* poly(A)+ RNA probed with radiolabelled *AtHAL3a* antisense riboprobe. S1 and S2, F_2 homozygous sense lines; WT, control wild type; AS1 and AS2, F_2 homozygous antisense lines.

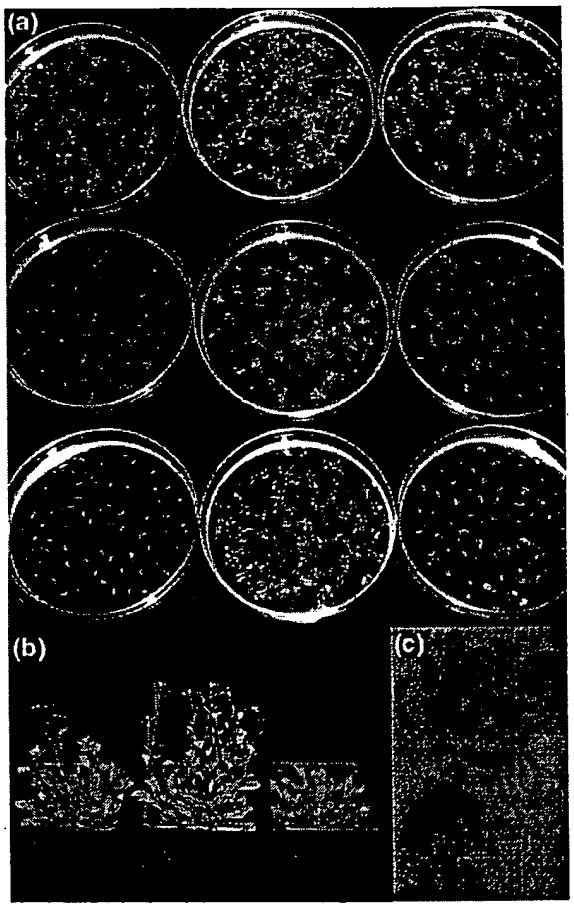


Figure 8. Effect of *AtHAL3a* constitutive expression on *Arabidopsis* stress tolerance and plant growth.
(a) *Arabidopsis* wild-type (left) and *AtHAL3a* transgenic F_2 homozygous lines sense S1 (centre) and antisense AS1 (right) on medium: MSS (top plates), MSS+100 mM NaCl (middle plates) and MSS+200 mM sorbitol (bottom plates), after 12 days' culture.
(b) *Arabidopsis* wild-type (left) and *AtHAL3a* transgenic F_2 homozygous lines sense S1 (centre) and antisense AS1 (right), after 40 days' culture.
(c) Closer view of plants of wild-type (left) and sense S1 (right) from (a) (middle plates).

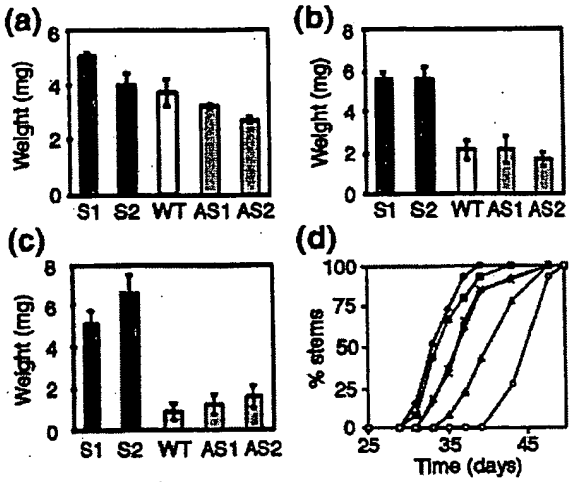


Figure 9. Growth comparison between *Arabidopsis* wild-type and *AtHAL3a* transgenic plants.
(a) Dry weight of plants on MSS media after 12 days' culture. S1 and S2, F_2 homozygous sense lines; WT, control wild type; AS1 and AS2, F_2 homozygous antisense lines. (b) As (a), but on MSS+100 mM NaCl. (c) As (a), but on MSS+200 mM sorbitol. (d) Comparative time course of stem production between F_2 homozygous sense lines S1 (◆) and S2 (■), control lines wild type (+) and F_2 homozygous transformed with the binary vector pBin19 (×), and F_2 homozygous antisense lines AS2 (Δ) and AS1 (○).
Each data point in (a–c) is the weight of 10 plants and corresponds to the mean value calculated from 50 plants of each line from three different experiments, and in (d) corresponds to the value, expressed in number of floral stems, calculated from 15 plants of each line. Error bars correspond to the standard error.

microsomal NADPH-cytochrome P_{450} reductase (CPR) (Wang *et al.*, 1997), along with AtHAL3a, do not show conserved polypeptide fragments in AtHAL3a encompassing the FMN binding site. However, the CPR structural X-ray determinations have showed that the FMN isoalloxazine ring-binding domain (five-stranded parallel β-sheets flanked by five α-helices) is approximately 170 amino acids long (Wang *et al.*, 1997), similar in length to the consensus domain shared by AtHAL3a and the prokaryote flavoprotein family (see arrows in Figure 2).

Transgenic Arabidopsis *overexpressing sense and anti-sense* AtHAL3a

Using *Agrobacterium*-mediated transfer, the *AtHAL3a* gene was stably integrated, in sense and antisense orientation, in the plant genome and expressed in the transgenic plants. F_1 plants were checked for transgene integration and *AtHAL3a* expression by PCR and Northern blot, respectively (not shown). Two sense (S1 and S2) and two antisense (AS1 and AS2) homozygous F_2 transgenic lines, that respectively showed increased and decreased *AtHAL3a* transcript amounts compared with the wild type, were chosen for further analysis (Figure 7).

Figures 8 and 9 show the effect of constitutive altered expression of *AtHAL3a* on *Arabidopsis* growth and stress tolerance. Plants that overexpress *AtHAL3a* gene show a faster growth rate than the wild type, while the *AtHAL3a* antisense plants produce the opposite phenotype (Figure 8a, top and Figure 9a). The differential growth rate observed is constant during plant development, particularly affecting the time course of floral stem production (Figures 8b and 9d). Sense plants also showed improved salt (Figure 8a, middle and Figure 9b) and osmotic (Figure 8a, bottom and Figure 9c) tolerance compared to wild-type and transgenic antisense plants. This improved tolerance is observed during germination and development. Sense transgenic plants developed roots and true leaves and continued growing under stress conditions, while wild-type plants mainly remained at the cotyledon stage (Figure 9c).

Discussion

In our search for plant genes homologous to the yeast halotolerance gene *HAL3*, we have identified a novel family of eukaryotic flavoproteins. Homology between yeast HAL3 and *Arabidopsis* AtHAL3a and AtHAL3b was restricted to a central domain of 180 amino acids, and database searches revealed a number of sequences from fungi, plants and animals which share the same domain. Its conservation through evolution points to a fundamental role in the physiology of eukaryotic cells. On the other hand, the acidic tail of fungal HAL3, mainly composed of glutamic and aspartic acid residues, is missing in the homologous proteins from plants and animals. The function of this acidic tail in the case of yeast HAL3 is not clear. It is important for sodium tolerance (Ferrando *et al.*, 1995) and to stimulate the growth of *sit4* mutants (Di Como *et al.*, 1995) but it is little required for lithium tolerance (present work). However, addition of the yeast acidic tail to AtHAL3a improves the complementation of yeast *hal3* mutants by the homologous plant protein. Yeast HAL3 is an inhibitory subunit of the protein phosphatase PPZ1 (Nadal *et al.*, 1998). It is plausible that the acidic tail participates in fostering the association of HAL3 with PPZ1, and that this reinforced association is required for some phenotypes of HAL3 but not for others, where a weaker interaction mediated by the conserved core could suffice. It would be interesting to determine whether plant and animal proteins homologous to yeast HAL3 interact with protein phosphatases related to yeast PPZ1.

In yeast, the protein phosphatase PPZ1 and its regulatory subunit HAL3 act as important determinants of salt tolerance by regulating the expression of the *ENA1* gene, encoding the major sodium extrusion pump of *S. cerevisiae* (Ferrando *et al.*, 1995; Nadal *et al.*, 1998). In addition, HAL3, probably via its interaction with PPZ1, also modulates the yeast cell cycle by regulating the expression of G1 cyclin genes (Di Como *et al.* 1995). A third function of the HAL3-PPZ1 regulatory complex is the modulation of the yeast MAP kinase pathway determining cell wall integrity (Nadal *et al.*, 1998). Therefore HAL3, probably by its interaction with the PPZ1 protein phosphatase, regulates genes concerned with ion homeostasis, cell cycle and cell wall integrity. Given the partial complementation of yeast *hal3* mutants by the *Arabidopsis* HAL3 homolog AtHAL3a, we speculate that plant (and probably animal) HAL3 homologous proteins may have similar mechanisms of action to the corresponding yeast gene. It could very well be that HAL3 proteins of plants and animals regulate, via a PPZ1-like protein phosphatase, the expression of genes related to the cell cycle, ion homeostasis and osmotic stability.

A bacterial family of flavoproteins also shows a significant homology to the conserved central domain of the eukaryotic homologous family. Interestingly, the homologous sheared region precisely expanded the eukaryotic conserved domain. These prokaryotic flavoproteins, like AtHAL3a, contain the flavin cofactor FMN. Because flavin coenzymes are involved in oxireduction reactions, an oxidoreductase activity has been suggested for these bacterial proteins, supposedly involved in an oxidative pathway of DNA synthesis and pantothenate metabolism (Spitzer and Weiss, 1985; Spitzer *et al.*, 1988). It would be interesting to investigate whether eukaryotic HAL3 homologous proteins experience redox changes, and if these changes influence their interaction with other proteins such as PPZ1.

Some clues for the physiological role of *Arabidopsis* HAL3 homologs may be provided by its pattern of expression. *AtHAL3a* is expressed at similar levels in most organs. The higher mRNA accumulation occurs in mature seeds. *AtHAL3b* follows the pattern of *AtHAL3a* expression, although its transcript levels were much lower (particularly in seeds). However, *AtHAL3b* was induced under salt stress to reach expression levels similar to *AtHAL3a*. The divergence in the 5′ upstream sequences between both genes probably reflects the differences in their expression.

In situ localization of mRNAs revealed that *AtHAL3a* expression was mainly restricted to the phloem part of vascular tissues. During embryogenesis, *AtHAL3a* mRNA is detected in the cotyledons and hypocotyl of mature seeds, mainly associated with embryo-specialized cell types from hypocotyl and cotyledon provascular tissues. This spatial pattern of expression during seed development is very similar to that shown by the late-embryogenesis-abundant genes maize *Rab28* (Niogret *et al.*, 1996), *Arabidopsis Atrab28* (Arenas-Mena *et al.*, 1999) and *Arabidopsis* peroxiredoxin antioxidant *AtPer1* (Haslekäs *et al.*, 1998). Since developing vascular centres play a major role in a variety of developmental processes (Nelson

and Langdale, 1992), and hormones and peptides are transported through the vascular system (Pearce *et al.*, 1991), this accumulation in cells of developing tissues and vascular structures is thought to be involved in late embryo-differentiation processes (Niogret *et al.*, 1996). AtHAL3a may well be involved in the cell cycle of developing vascular tissues, while in mature phloem it could be part of a signal-transduction pathway for defence against osmotic stress.

Transgenic *Arabidopsis* plants constitutively over-expressing *AtHAL3a* showed improved growth and salt and osmotic tolerance. The antisense plants, despite their altered growth phenotype, do not show any stress-hypersensitive response. These phenotypes could well be a consequence of the strength of AtHAL3a control on the expression of genes related to the cell cycle, ion homeostasis and osmotic stability during embryogenesis and in the phloem of vegetative tissues. Indeed, the fact that gain and loss of AtHAL3a activity correlate with the growth rate of *Arabidopsis* plants points to an important rate-limiting role in developmental growth.

It is expected that these novel flavoproteins will be implicated in many plant processes. Biochemical and X-ray analysis are in progress to unravel the possible redox activity and the structure of AtHAL3a, in order to gain more insight into the mechanisms of action of these new plant flavoproteins.

Experimental procedures

Plant material and stress treatments

Arabidopsis thaliana ecotype Columbia was grown in the greenhouse at 25°C under 8 h dark, 16 h light. For seedling stress, wild-type and transgenic surface-sterilized *Arabidopsis* seeds were sown in Petri dishes containing 25 ml MSS medium [MS (Murashige and Skoog, 1962) + 3% sucrose], MSS+100 mM NaCl for salt stress, MSS+200 mM sorbitol for osmotic stress, and MSS medium supplemented with 10 μM ABA (48 h after day 12) for ABA treatments. Seedlings were grown for 12 days at 25°C under fluorescent light, 8 h dark and 16 h light.

Isolation of genomic clones

Approximately 50 000 plaque-forming units, from an EMBL3 library of *A. thaliana* DNA partially digested with *Sau*3A (Clontech, Palo Alto, CA, USA), were screened with the *AtHAL3a* cDNA (Stock 164P17T7) obtained from the Arabidopsis Biological Resource Centre (Ohio State University, Columbus, OH, USA). After washing at low stringency (55°C in 2 × SSC, 0.5% SDS), five positive clones were isolated that hybridized with *AtHAL3a*. The genomic clones were mapped with different restriction enzymes and were shown to be not identical. Three of these clones contained *AtHAL3a* and the other two contained *AtHAL3b*. Genomic clones AG1 and AG5, which contain *AtHAL3a* and *AtHAL3b* coding regions, respectively, were selected for further analysis.

Isolation of cDNA clones

300 000 plaque-forming units from an *A. thaliana* Uni-ZAP XR library (Stratagene, La Jolla, CA, USA), constructed from 4-week-old adult plants grown under long-day conditions, were also screened with the *AtHAL3a* cDNA (Stock 164P17T7). After washing at low stringency (2 × SSC, 0.5% SDS, at 55°C), nine positive cDNA clones were obtained, all of which contained *AtHAL3a*. cDNA clone AC8, with the complete *AtHAL3a* coding sequence, was selected for subsequent experiments. AC8 cDNA sequences can be deduced joining nucleotides 888–1289 and 1407–1867 from accession number AF166262.

Plasmids, yeast strains and culture conditions

Yeast strains used in this study were RS16 (wild type) (Gaxiola *et al.*, 1992) and RS48 (RS16 *hal3::LEU2*) (Ferrando *et al.*, 1995). Transformants were obtained using the lithium acetate procedure (Ito *et al.*, 1983). Cells were routinely grown in YPD medium (1% yeast extract, 2% Bacto peptone and 2% glucose). Transformants were selected by plating on minimal medium (SD) containing 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco) and 50 mM MES adjusted to pH 5.5 with Tris. Salt tolerance was determined in solid YPD medium (containing 2% bacteriological grade agar) supplemented with NaCl and LiCl to the indicated final concentrations by a drop assay. The *AtHAL3a* coding sequence was PCR amplified from AC8 adding extra 5′-end *Hin*dIII and 3′-end *Bam*HI restriction sites and cloned *Hin*dIII/*Bam*HI into pBluescript SK+ (Stratagene) creating plasmid pA8. Then it was cloned as a 630 bp *Xho*I fragment, PCR amplified from pA8 creating extra *Xho*I restriction sites at both ends, in the appropriated orientation, into yeast vector pRS699 (Serrano and Villalba, 1995) which contains the strong constitutive *PMA1* promoter, creating pRS699 *AtHAL3a*. The *HAL3* coding region was introduced into pRS699, as described above for *AtHAL3a*, as a 1.7 kb *Xho*I fragment from plasmid pA44 (*HAL3* coding region, cloned *Eco*RI/*Bam*HI in vector pUC19, New England Biolabs), to produce pRS699 *HAL3*. The 1.4 kb *Eco*RI/*Kpn*I fragment from pA44, which contains *HAL3* coding region lacking the acidic tail, was cloned into yeast vector pYES 2.0 (Invitrogen, CA, USA), creating pA308. The 1.4 kb *Xba*I/*Hin*dIII fragment from pA308, which adds an extra *Xho*I site at the 5′ end, was subcloned into vector pBluescript SK(+) (Stratagene), creating pA321. The 1.4 kb *Xho*I fragment from pA321 was then cloned, in the appropriated orientation, into yeast vector pRS699 to produce pRS699 *HAL3Δ*. A chimeric gene containing *AtHAL3a* coding region plus *HAL3* acidic tail was constructed appending the 0.63 kb *Hin*dIII/*Kpn*I fragment from plasmid pA8 (containing *AtHAL3a* coding sequence) and the 0.24 kb *Kpn*I/*Pst*I fragment (containing *HAL3* acidic tail) isolated from plasmid pA44 and then cloned into *Hin*dIII/*Pst*I vector pBluescript SK(+), creating pA348. The chimera was finally isolated from pA348 by PCR adding extra *Xho*I sites at both ends and cloned as a 0.9 kb *Xho*I fragment, in the appropriate orientation, into yeast vector pRS699 to produce pRS699 *AtHAL3*+ac tail.

Sequencing

PCR amplified DNA was sequenced for detection of possible mistakes. Sequence on both strands was determined according to Sanger *et al.* (1977) by double-stranded plasmid sequencing in pBluescript using Sequenase (United States Biochemicals). Sequence analysis was performed using the WISCONSIN Package

version 9.0 (Genetics Computer Group, Madison, Wisconsin, USA). Genomic *AtHAL3a and AtHAL3b* nucleotide sequences have been submitted to the Genebank/EMBL Databank under the accession numbers AF166262 and AF166263, respectively.

DNA gel blotting, Northern blots and hybridization

Genomic DNA gel blots and Northern analysis were performed using approximately 10 µg DNA and 5 µg poly(A)+ RNA per track, respectively. Isolated DNA fragments were nick-translated in the presence of α-[^{32}P]dCTP to be used as probes (Maniatis *et al.*, 1982). α-[^{32}P]CTP radiolabelled RNA probes were performed according to the manufacturer's instructions (Boehringer Mannheim). Probe *AtHAL3a* cDNA, was a 0.6 kb *Hin*dIII/*Bam*HI fragment from plasmid pA8; probes *AtHAL3a* and *AtHAL3b* 3'-untranslated, were two 180 and 200 bp *Hin*dIII/*Bam*HI fragments obtained by PCR amplification from genomic clones AG1 and AG5 3'-untranslated region, and to which extra 5'-end *Hin*dIII and 3'-end *Bam*HI restriction sites were added, respectively. *AtHAL3a* riboprobe was transcribed from linear *Hin*dIII pA8 using T3 RNA polymerase. Hybridization was performed in PSE (0.3 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA) at 65°C for Southern and at 55°C for Northern and in PSE+50% formamide at 55°C when RNA probe was used. Filters were washed at high stringency (0.1 × SSC, 0.5% SDS at 65°C).

In situ *hybridization*

For *in situ* hybridization, digoxigenin-labelled RNA probes were prepared according to the manufacturer's instructions (Boehringer Mannheim) and performed as previously described (Goday *et al.*, 1994). Sense and antisense probes were transcribed from *Bam*HI and *Hin*dIII linear pA8 using T7 and T3 RNA polymerases, respectively. In all cases, no signal over background was observed using control sense strand probes.

Chimeric AtHAL3a protein synthesis and purification

The full length of *AtHAL3a* coding region was PCR amplified from cDNA clone AC8 adding extra 5'-end *Bam*HI and 3'-end *Hin*dIII restriction sites and subcloned *Bam*HI/*Hin*dIII into vector pET-28a(+) (Novagen, MA, USA), overexpressed in *E. coli* and purified by affinity chromatography with His.Bind metal chelation resin (pET System, Novagen). Total protein yield was 5 mg. Protein extraction and electrophoresis were performed as previously described (Niogret *et al.*, 1996).

Preparation of extracts and HPLC analysis

To release the fluorescent chromophore, 0.5 ml from the purified enzyme fraction 6 were treated with perchloric acid (5% final concentration) at 0°C for 15 min. Protein was clarified by centrifugation at 2000 r.p.m. (1000 *g*) for 5 min and further treated as previously described (Murguía *et al.*, 1995, 1996). 10 ml of extract were analysed by HPLC in a Waters 600 E liquid chromatograph. Samples were injected onto a reversed-phase C18 column (Nova-Pak, 4 × 250 mm, 4 µm particle size, Waters) maintained at 25°C and equilibrated in 4% MeOH and 83.3 mM triethylammonium phosphate (pH 6.0) according to Lim (1991). After injection, a gradient of MeOH (4–100%), with a flow rate of 1 ml min^{-1}, was applied over 20 min. FAD, FMN and riboflavin were detected in a Waters 486 absorbance detector as described by Lim (1991). Peaks were identified by co-injection with standard. Peak areas were quantified with a Waters 746 integrator by comparison with known amounts of FAD, FMN and riboflavin standards.

Plant transformation

The *AtHAL3a* coding region was isolated by PCR from plasmid pA8, and cloned in sense and antisense orientation as a 0.63 kb *Hin*dIII/*Bam*HI fragment into plasmid pJIT 163 (Guerineau *et al.*, 1992), creating pA18 and pA61, respectively. The 2.1 kb *Kpn*I/*Xho*I DNA fragment from pA18 and pA61, which contains the *AtHAL3a* gene flanked by a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer and by the CaMV polyadenylation sequence, was finally cloned into the binary plant vector pBin19 (Bevan, 1984) creating pA31 and pA76, respectively. *Agrobacterium* helper strain LBA 4404 (Hoekema *et al.*, 1983), was transformed with pA31 or pA76 by high-voltage electroporation (Wen-Jun and Forde, 1989), and used for plant transformation. *Arabidopsis thaliana* adult plants (5 weeks old) were agroinfected by infiltration (Bechtold *et al.*, 1993) and grown in the greenhouse to collect seeds. F_0 seeds were grown in MSS medium supplemented with 50 mg ml^{-1} kanamycin (K4378, Sigma Chemical Company, MO, USA). A yield of one transformant per 1000 seeds was obtained. Ten independent kanamycin resistant F_0 plants were selected for each transformation, transferred to soil after 15 days and grown in greenhouse to collect seeds. F_1 plants were checked for transgene integration and *AtHAL3a* expression by PCR and Northern blot, respectively. Two lines for each transformation were selected which showed higher and lower expression of *AtHAL3a*, respectively, and which segregated 3 : 1 in kanamycin, as expected for a single integration of the construction in the plant genome. From each of the selected lines, 10 F_1 plants were grown and seeds were collected. F_2 plants were segregated in MSS media supplemented with 50 mg ml^{-1} kanamycin. One homozygous line was selected for each sense and anti-sense F_2, named S1, S2 and AS1, AS2, respectively, and used for phenotype characterization and stress treatments.

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GenBank accession numbers AtHAL3a (AF166262) and AtHAL3b (AF166263).

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RESEARCH PAPER

Overexpression of *NtHAL3* genes confers increased levels of proline biosynthesis and the enhancement of salt tolerance in cultured tobacco cells

Ikuko Yonamine, Kazuya Yoshida*, Keiji Kido, Atsushi Nakagawa, Hideki Nakayama and Atsuhiko Shinmyo

Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), 8916-5 Takayama-cho, Ikoma-shi, Nara 630-0101, Japan

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Abstract

The Hal3 protein of *Saccharomyces cerevisiae* inhibits the activity of PPZ1 type-1 protein phosphatases and functions as a regulator of salt tolerance and cell cycle control. In plants, two *HAL3* homologue genes in *Arabidopsis thaliana*, *AtHAL3a* and *AtHAl3b*, have been isolated and the function of *AtHAL3a* has been investigated through the use of transgenic plants. Expressions of both *AtHAL3* genes are induced by salt stress. *AtHAL3a* overexpressing transgenic plants exhibit improved salt and sorbitol tolerance. *In vitro* studies have demonstrated that AtHAL3 protein possessed 4′-phosphopantothenoylcysteine decarboxylase activity. This result suggests that the molecular function of plant *HAL3* genes is different from that of yeast *HAL3*. To understand the function of plant *HAL3* genes in salt tolerance more clearly, three tobacco *HAL3* genes, *NtHAL3a*, *NtHAL3b*, and *NtHAL3c*, from *Nicotiana tabacum* were identified. *NtHAL3* genes were constitutively expressed in all organs and under all conditions of stress examined. Overexpression of *NtHAL3a* improved salt, osmotic, and lithium tolerance in cultured tobacco cells. *NtHAL3* genes could complement the temperature-sensitive mutation in the *E. coli dfp* gene encoding 4′-phosphopantothenoyl-cysteine decarboxylase in the coenzyme A biosynthetic pathway. Cells overexpressing *NtHAL3a* had an increased intracellular ratio of proline. Taken together, these results suggest that NtHAL3 proteins are involved in the coenzyme A biosynthetic pathway in tobacco cells.

Key words: *HAL3* genes, HAL3 proteins, overexpression, proline biosynthesis, salt tolerance, tobacco cells.

Introduction

The *HAL3/SIS2* gene of *Saccharomyces cerevisiae* was identified by its ability to confer salt tolerance to wild-type cells in the presence of toxic concentrations of sodium chloride. Intracellular levels of sodium and potassium were dependent on the level of *HAL3* expression. Expression of *ENA1/PMR2A*, a gene encoding the plasma membrane Na^+-ATPase involved in sodium and lithium efflux, is negatively regulated by both the Hal3 and Ppz1 signal transduction pathways, and positively regulated by a calcineurin-dependent pathway. Hal3 protein directly interacts with Ppz1 protein and inhibits its protein phosphatase activity (Mendoza *et al.*, 1994; Posas *et al.*, 1995; Ferrando *et al.*, 1995; Marquez and Serrano, 1996; de Nadal *et al.*, 1998). It has also been reported that overexpression of *HAL3/SIS2* suppressed the growth defect and stimulated G1 cyclin expression in a type-2A protein phosphatase *sit4* mutant (Di Como *et al.*, 1995; Sutton *et al.*, 1991; Fernandez-Sarabia *et al.*, 1992). These data suggest that Hal3 protein is a multifunctional regulator involved in salt tolerance, cell cycle control and cell wall integrity via its interaction with the regulatory subunit of Ppz1 protein (de Nadal *et al.*, 1998; Clotet *et al.*, 1999). It has also been suggested that these regulatory activities are dependent on intracellular K^+ concentration and pH which are mediated by a potassium transport system involving the Trk1 and Trk2 proteins (Yenush *et al.*, 2002).

In plants, two *HAL3* homologue genes were isolated from *Arabidopsis thaliana* and characterized. The expression of these genes is increased under conditions of salt stress. Transgenic *Arabidopsis* overexpressing the *AtHAL3a* gene showed improved tolerance to salt and

* To whom correspondence should be addressed. Fax: +81 743 72 5469. E-mail: kazz@bs.aist-nara.ac.jp

osmotic stress (Espinosa-Ruiz *et al.*, 1999). Although these data suggest some relationships between the *AtHAL3a* gene and osmolyte accumulation and/or transport of toxic sodium ions, the molecular function of plant HAL3 protein in salt tolerance is still unknown. Recently, it was reported that AtHAL3a protein containing a flavin mononucleotide catalysed the reaction from the decarboxylation of 4′-phosphopantothenoylcysteine to 4′-phosphopantetheine *in vitro* (Espinosa-Ruiz *et al.*, 1999; Kupke *et al.*, 2001). This reaction is known to be involved in the coenzyme A biosynthesis pathway of *E. coli* (Kupke *et al.*, 2000; Begley *et al.*, 2001). Kupke *et al.* (2001) suggest that the AtHAL3a protein functions in the coenzyme A biosynthetic pathway of plants, and is not involved in signal transduction like the yeast Hal3 protein. To investigate this hypothesis, a cultured tobacco cell system was chosen to examine the metabolic change in cells caused by the overexpression of plant *HAL3*. *Nicotiana tabacum* L.cv. Bright Yellow-2 (BY2) cells are fast growing, highly homogenous (Nagata *et al.*, 1992) and can be easily transformed. Since it is considered that the homogenous cell culture system is suitable for the analysis of metabolic change in plant cells, BY2 cells were chosen to investigate the change in amino acid levels caused by the overexpression of *HAL3* gene(s).

In this report, three *HAL3* homologues were isolated from *N. tabacum*. The *NtHAL3* genes were constitutively expressed in all organs examined, regardless of the conditions of stress in the tobacco plant. Salt, hyperosmotic, and lithium tolerance were improved by overexpression of *NtHAL3a* in BY2 cells. This indicates that the *NtHAL3a* gene is functionally homologous to *AtHAL3a*. It is also reported that the *NtHAL3* gene could complement the temperature-sensitive *dfp* mutation of *E. coli*, and proline levels were increased in *NtHAL3a* overexpressing BY2 cells.

Materials and methods

Plant materials, culture and transformation

N. tabacum L. cv. SR-1 was grown in soil in a temperature-controlled greenhouse at 25 °C with a 16/8h light/dark cycle. Culture conditions and transformation of cultured tobacco BY2 cells has been described previously (Nakayama *et al.*, 2000).

Isolation of NtHAL3 *cDNAs*

Total RNA was extracted from exponentially growing BY2 cells and from the shoot apex of tobacco SR-1. Poly (A) RNA was purified by the Oligotex-dT30 kit (Takara Bio Ink, Otsu, Japan), and cDNA libraries were constructed in the ZIP-LOX vector (GibcoBRL, Rockville, MD, USA). Plaques were replicated on nylon membranes (Hybond-N$^+$, Amersham Pharmacia, Piscataway, NJ, USA), and hybridization was performed at 65 °C in buffer as described previously (Church and Gilbert, 1984). The BY2 cDNA library was screened with a ^{32}P-labelled partial fragment of *AtHAL3* cDNA (40–594 bp, accession no. U80192, 1997), and five positive clones were isolated from the BY2 cDNA library. The SR-1 cDNA library was subsequently screened with ^{32}P-labelled *NtHAL3a* cDNA that was isolated from BY2, and 11 positive clones were isolated.

Detection of mRNA expression of the NtHAL3 *genes*

Total RNA was extracted from each sample (various organs of 4–6-week-old tobacco) as previously described for Northern hybridization (Nakayama *et al.*, 2000) or by the AGPC method for RT-PCR (Chomczynski *et al.*, 1987). For RT-PCR analysis, first-strand cDNA was synthesized from 1 μg of total RNA with a first-strand cDNA kit (Perkin Elmer, Branchburg, NJ, USA), and was subsequently used as the template for the PCR reaction. PCR was performed with the following cycle parameters using specific primer sets; once at 94 °C for 2 min; 29 and 31 cycles for *NtHAL3a* and *NtHAL3c* or 32 and 34 cycles for *NtHAL3b* at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and once at 72 °C for 5 min. The specific primer sets are NtHAL3a (5′-CAGAGATGGAACCGGTTCAG-ATT-3′ and 5′-GCGTCATAATAGAGTCTTACAGCTTGGA-3′), *NtHAL3b* (5′-GGTGCAGTAAAGAATCCTTTTCGATG-3′ and 5′-CATGACCTGTGGATCACGA-3′), or *NtHAL3c* (5′-GTCCTGTT-CGTTCCGTCGA-3′ and 5′-GTGCGTCATAATAGATTCTTACA-GCTTGAT-3′). Actin was used as an internal control, and the set was 5′-CCTCTTAACCCGAAGGCTAA-3′and 5′-GAAGGTTGG-AAAAGGACTTC-3′. PCR reaction was performed using the following parameters; once at 94 °C for 2 min; 28 and 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and once at 72 °C for 5 min.

Complementation test with a mutant strain of E. coli

For the complementation test with an *E. coli* mutant, the *NtHAL3* coding sequences flanked by *Bam*HI and *Xho*I sites were cloned using the *Bgl*II and *Xho*I cloning sites of pKC7 (Rao and Rogers, 1979). *NtHAL3* mutated sequences flanked by *Eco*RI and *Sac*I sites were cloned using the *Eco*RI and *Sac*I cloning sites of each pZL1::*NtHAL3*cds plasmids, and subsequently these plasmids were used as the next PCR template to amplify the mutated *NtHAL3* sequences flanked by *Eco*RI and *Xho*I sites. The *NtHAL3* mutated sequences were cloned using the *Eco*RI and *Xho*I cloning sites of pKC7. Primer set for *NtHAL3* coding sequences; *NtHAL3a* (a-*Bam*HI-F, 5′-CGGGATCCATGGAGACTTCAGAGATGG-3′ and a,c-*Xho*I-R, 5′-AATCTCGAGTCACGCCACGTTGCTG-3′), *NtHAL3b* (b,c- *Bam*HI-F, 5′-CGGGATCCATGGAGCCTATGA-CTTCAGAG-3′and b-*Xho*I-R, 5′-CCGCTCGAGTCACGACACG-TTGCTGCC-3′) or *NtHAL3c* (b,c-*Bam*HI-F and a,c-*Xho*I-R). Mutagenesis primer sets for active-site mutation; *NtHAL3a* (a-*Eco*RI-F, 5′-CCGGAATTCATGGAGACTTCAGAGATGG-3′ and muHN-*Sac*I-R, 5′-CTCCGGAGCTCGATGTTTAG-3′), *NtHAL3b* (b,c-*Eco*RI-F, 5′-GGAATTCATGGAGCCTATGACTT and muHN-*Sac*I-R), *NtHAL3c* (b,c-*Eco*RI-F and muHN-*Sac*I-R). The constructs were introduced into the *E. coli* temperature-sensitive *dfp* mutant strain BW369 (Spitzer and Weiss, 1985). Transformants were cultured on solid LB Amp plates at 30 °C for 24 h or 42 °C for 12 h to examine complementation of the *dfp* mutation.

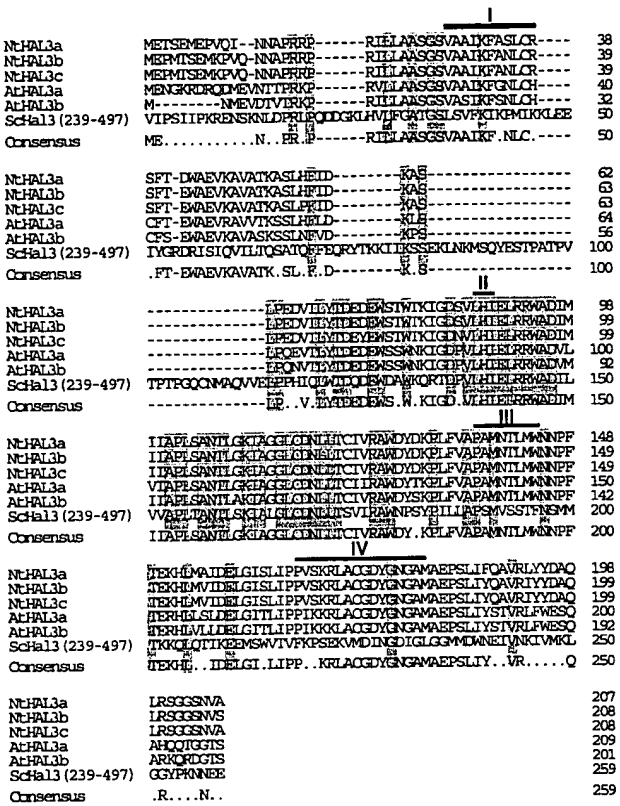
Construction of binary plasmids

Full-length *NtHAL3a* cDNA in pZL1 was digested with *Sal*I and *Not*I, and subsequently cloned into the *Xho*I and *Not*I cloning sites of pMS1 which is removed by the hygromycin resistance gene from pMSH1 (Kawasaki *et al.*, 1999).

Analysis of stress tolerance

6-day-old BY2 cells were harvested in a 50 ml centrifuge tube and centrifuged for 5 min at 800 *g*. After removal of the culture medium, the cell density was adjusted to 50% (v/v) with fresh medium. Five millilitres of cells were transferred to a 300 ml Erlenmeyer flask containing 95 ml of modified LS medium ($CaCl_2.2H_2O$, 0.3 mM)

a



b

	NtHAL3a	NtHAL3b	NtHAL3c
AtHAL3a	72	73	72
AtHAL3b	72	74	73
NtHAL3b	95	—	—
NtHAL3c	94	98	—

Fig. 1. Comparison of deduced amino acid sequences among various HAL3 proteins. (a) Comparison is shown between the deduced amino acid (aa) sequences of tobacco NtHAL3a (207 aa), NtHAL3b (208 aa), and NtHAL3c (208 aa); *Arabidopsis* AtHAL3a (209 aa) and AtHAL3b (201 aa); and yeast Hal3 (259 aa of conserved region). Roman numerals indicate the substrate binding helix (I); inserted His motif (II); PXMNXXMW motif (III); substrate recognition clamp (IV) of *P*PC-DC (Kupke *et al.*, 2001). The amino acid sequence alignment was performed with Gene Works multiple sequence alignment software (Oxford Molecular Group Inc., Campbell, CA). (b) Percentage identity of the predicted amino acid sequences among plant HAL3 proteins including the three NtHal3s and two AtHal3s.

containing 100 μg ml^{-1} kanamycin and 250 μg ml^{-1} carbenicillin with 100 or 140 mM NaCl for salt stress, 30 or 60 mM LiCl for Li^+ stress, 150 or 300 mM sorbitol for osmotic stress for 5 d.

Measurement of amino acid content in BY2

Total free amino acids were extracted from 1 g fresh weight of cells grown in a no-stress medium for 5 d. Cells were homogenized in liquid nitrogen in 5 ml of a methanol:chloroform:water mixture (12:5:2, by vol.) following previously described methods (Nakayama *et al.*, 2000). After centrifugation at 2300 *g* for 5 min, the supernatant was collected in a 50 ml centrifuge tube and the extraction procedure was repeated twice. 10 ml of chloroform and 5 ml of water were added to the pooled extracts and mixed vigorously before being centrifuged at 2300 *g* for 5 min. The aqueous layer was collected in a 50 ml centrifuge tube and the organic layer was re-extracted with an additional 5 ml of water. The pooled aqueous layer was evaporated at 80 °C for 2 d and then dissolved in 0.8 ml of water and filtered through centrifugal filter units (0.2 μm pore; Ultrafree-MC, Nihon Milipore Ltd., Tokyo, Japan). Amino acid analysis was performed with an amino acid analyser (Model L-8500, Hitachi Ltd., Tokyo, Japan) with ninhydrin reaction of samples.

Results

Isolation of NtHAL3 *cDNAs*

To isolate tobacco *HAL3* homologues, a tobacco BY2 cDNA library was screened with a partial *AtHAL3a* cDNA

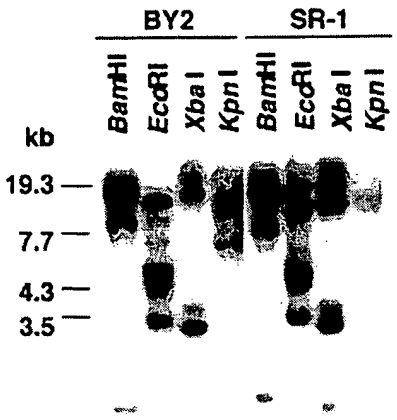


Fig. 2. Genomic Southern blot analysis of the *NtHAL3* gene. 30 μg each of genomic DNA of BY2 cells and tobacco leaves from SR-1 were digested with *Bam*HI, *Eco*RI, *Xba*I, or *Kpn*I, separated on 1.0% (w/v) agarose gel, and blotted onto nylon membrane. Membrane was hybridized with a ^{32}P-labelled cDNA fragment of *NtHAL3a* coding region at 65 °C in buffer as previously described (Church and Gilbert, 1984). The molecular size (kb) estimated by DNA size marker is indicated on the left side of the gel.

fragment as a probe. Out of approximately 400 000 plaques, five positive cDNA clones were isolated. The sequence of the longest cDNA showed homology with that of the *AtHAL3a* cDNA used as probe; this cDNA was named *NtHAL3a*. The *NtHAL3a* gene encodes a protein with 207 amino acid residues with a predicted molecular mass of 22.8 kDa and theoretical pI of 4.99 (Fig. 1a). Southern blot analysis suggested the presence of additional *HAL3* homologues in BY2 cells and the tobacco plant SR-1 (Fig. 2). Therefore, the BY2 cDNA library was re-screened, but no other *HAL3* homologues were obtained. In order to characterize these putative *HAL3* homologues further, a cDNA library derived from SR-1 shoot apex cells was screened with the cDNA fragment of *NtHAL3a* coding region as a probe. Eleven positive clones were isolated from approximately 200 000 plaques. Three of these clones were identical in sequences to that of the previously isolated *NtHAL3a* cDNA. The additional cDNA clones contained two different *HAL3* homologues; these genes were named *NtHAL3b* and *NtHAL3c*, respectively. Both genes encode proteins of 208 amino acids with predicted molecular masses of 23 kDa, but the theoretical pI of NtHAL3b and NtHAL3c proteins were 5.51 and 5.61, respectively (Fig. 1a). NtHAL3 proteins contain the conserved domain present in the middle region of yeast Hal3 protein (Leu-353 to Asn-435), but the aspartate- and glutamate-rich region in the carboxyl terminus of yeast Hal3 is not present in NtHAL3 (Fig. 1a). The predicted amino acid sequences of tobacco and *Arabidopsis* HAL3 proteins demonstrate a high degree of homology (Fig. 1b).

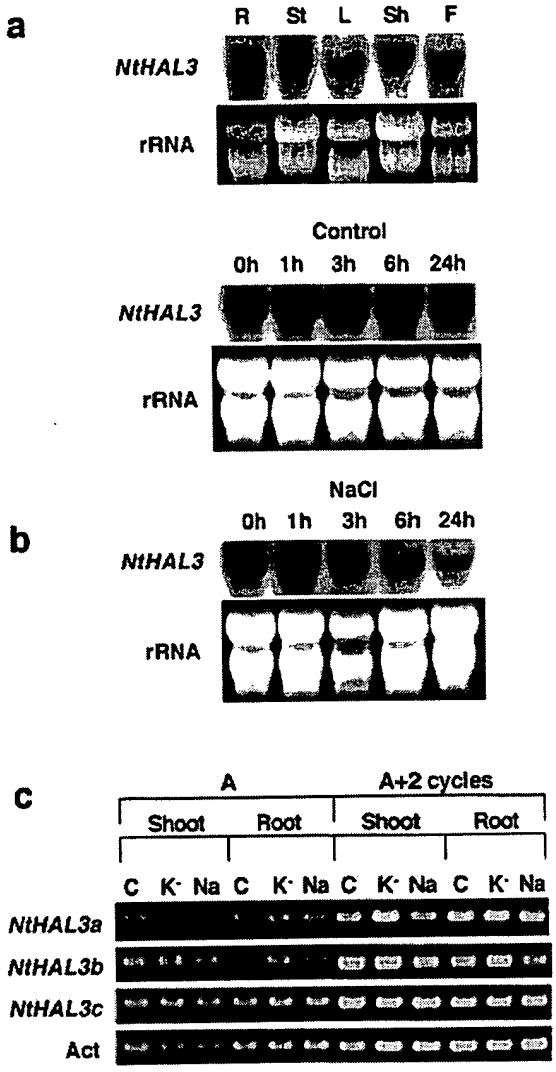


Fig. 3. Expression of the *NtHAL3* genes in tobacco plants. Distribution of *NtHAL3* mRNAs (a) and time-course of *NtHAL3* mRNA following 100 mM NaCl treatment (b) were investigated by northern blot analysis. 20 μg of total RNA was separated on a 1.0% (w/v) formaldehyde agarose gel and blotted onto nylon membranes. The membrane was hybridized with a ^{32}P-labelled cDNA fragment of *NtHAL3a* at 65 °C. rRNA was used as the internal control. Abbreviations: R, root; St, stem; L, young leaf; Sh, shoot; F, flower. Transcripts of the individual *NtHAL3* genes in the shoot and root under potassium depletion or sodium-stress conditions (c) were detected by RT-PCR. RNA samples were isolated from 7-d-old seedlings after 24 h incubation in MS liquid medium as control (C), potassium free medium (K–) or medium containing 100 mM NaCl (Na). The actin gene was used as an internal control (Act). A+2 cycles of PCR reactions showed that DNA amplification in reaction A was not saturated.

Expression of the NtHAL3 *genes*

To investigate the mode of *NtHAL3* expression, northern blot analysis was performed using the coding region of *NtHAL3a* cDNA as a probe. The presence of *NtHAL3* mRNAs was observed in all organs of the tobacco plant,

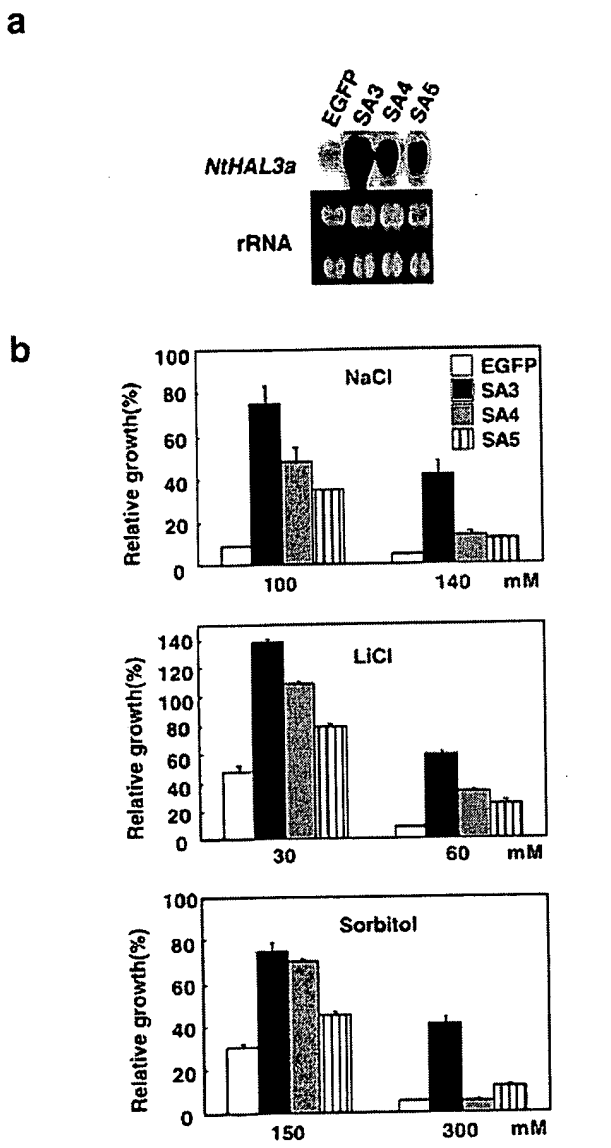


Fig. 4. Examination of growth effects of *NtHAL3a* overexpressing BY2 cells in medium containing NaCl, LiCl, or sorbitol. (a) mRNA level of *NtHAL3a* in the transgenic BY2 cells was detected by northern blot analysis. RNA was isolated from 5-d-old transgenic BY2 clones harbouring the *EGFP* gene (control) and three independent clones harbouring the *NtHAL3a* gene (SA3, SA4, SA5). Each lane was charged with 20 μg of total RNA, and hybridized with a ^{32}P-labelled cDNA fragment of *NtHAL3a*. rRNA was used as an internal control. (b) Each transgenic BY2 clone was cultured in liquid medium containing the indicated concentration of NaCl, LiCl, or sorbitol for 5 d. Cell growth was measured by the fresh weight of the cells, and the growth of each clone under the indicated stress conditions was calculated relative to growth under non-stress conditions. Error bars represent ±SD (n=3).

including roots, stem, young leaves, the shoot apex, and flowers (Fig. 3a). The level of *NtHAL3* mRNA did not change from 1–24 h after 100 mM NaCl treatment (Fig. 3b). Northern analysis detected the total mRNA derived from all three *NtHAL3* genes because the coding sequence of *NtHAL3a* was used as a probe. The coding sequence of the *NtHAL3* cDNAs are more than 96% identical, but are less than 29% identical in their 5′ untranslated regions. Although northern analysis was carried out with a DNA probe corresponding to the 5′ untranslated sequence of each gene, the mRNA signal of each *NtHAL3* was not clearly identified. In order to identify the expression patterns for each *NtHAL3* gene under stress conditions, RT-PCR analysis was performed with primer pairs specific for the DNA sequence corresponding to the 5′ untranslated regions and the coding region of *NtHAL3b* and *NtHAL3c*, and to the coding region of the *NtHAL3a*. The amount of detected *NtHAL3* isoform mRNA did not change after treatment of cells with 100 mM NaCl or a shortage of potassium (Fig. 3c).

Overexpression of NtHAL3a *improved salt, LiCl, and sorbitol stress tolerance in BY2 cells*

To investigate the molecular function of plant *HAL3* genes using the transgenic BY2 system, *NtHAL3a* was placed under the control of the CaMV 35S promoter and introduced into BY2 cells by *A. tumefaciens*-mediated transformation. Approximately 80 kanamycin-resistant calli were isolated, and some calli were checked for integration and expression of *NtHAL3a* by genomic PCR and northern blot analysis, respectively (data not shown). Expression of *NtHAL3a* varied widely, and three clones were selected for further analysis (SA3, SA4, and SA5; Fig. 4a). Growth inhibition of transgenic BY2 clones by salt stress, at 100 or 140 mM NaCl was observed. Although the growth of control transformed cells (EGFP) was markedly inhibited by NaCl stress, overexpression of *NtHAL3a* reduced the growth inhibition in direct relation to the level of *NtHAL3a* expression in transgenic BY2 cells (Fig. 4b). High salt concentrations can affect cell growth negatively through hyperostmotic stress and/or direct toxicity of the sodium ion. In order to identify which aspect of salt stress *NtHAL3* overexpression was affecting, growth inhibition experiments were carried out under hyperosmotic conditions with sorbitol and sodium-ion stress conditions with LiCl as a more toxic analogue of sodium (Serrano, 1996). The reduction of growth inhibition by LiCl and sorbitol stress was observed in *NtHAL3a* expressing BY2 cells (Fig. 4b). These results indicate that NtHAL3a relates to both hyper-osmotic stress and sodium ion toxicity in tobacco cells.

NtHAL3 *genes complement the* E. coli dfp *mutation*

The predicted amino acid sequences of the three NtHAL3 proteins contain four highly conserved motifs (I–IV; Fig. 1a) that form a domain of 4′-phosphopantothenoylcysteine decarboxylase (*P*PC-DC). These motifs are also found in AtHAL3a protein, and *P*PC-DC activity of AtHAL3a was shown biochemically by an *in vitro* study

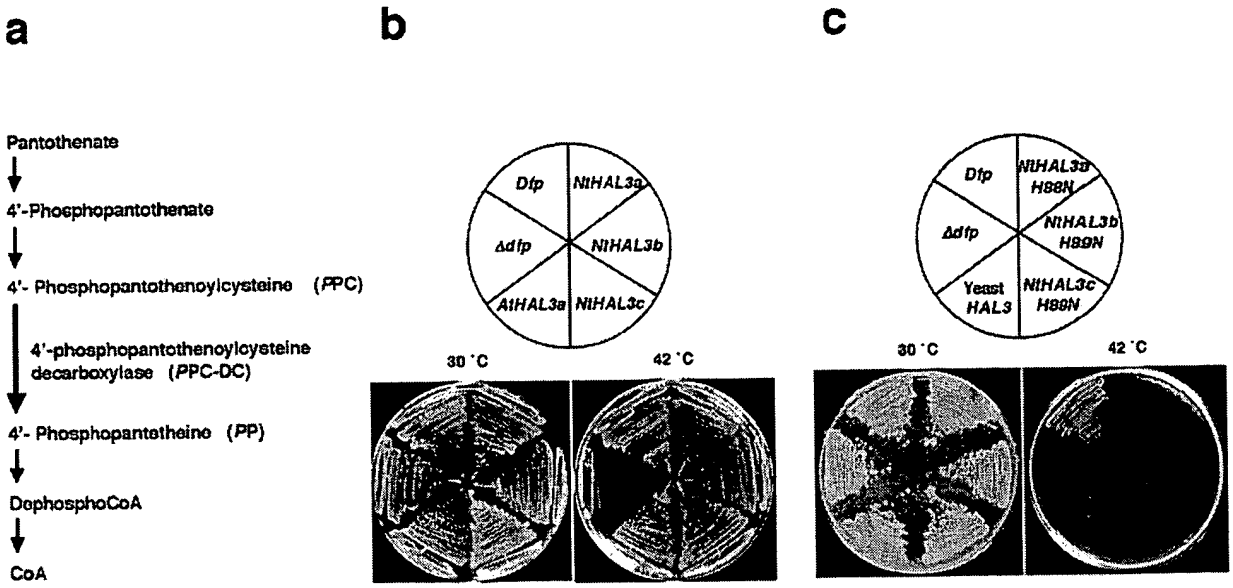


Fig. 5. Complementation analysis of *NtHAL3* genes with the temperature-sensitive *dfp* mutant of *E. coli*. (a) The coenzyme A biosynthetic pathway in *E. coli* (Begley *et al.*, 2001). (b, c) Strain BW369 (*dfp*-707) of *E. coli* was transformed with an expression vector containing each *NtHAL3* gene and the *AtHAL3* gene (b), and the active site mutant genes of *NtHAL3* and the yeast *HAL3* gene (c). Dfp is a positive control with the *E. coli dfp* gene and Δdfp is a negative control with an empty vector. Transformants were grown on LB Amp plates at 30 °C for 24 h or 42 °C for 12 h.

(Kupke *et al.*, 2001). *P*PC-DC is encoded by the *dfp* gene in *E. coli* and plays an important role in the coenzyme A biosynthetic pathway (Fig. 5a; Kupke *et al.*, 2000; Kupke 2001; Strauss *et al.*, 2001). To investigate whether NtHAL3 protein has *P*PC-DC activity, the ability of *NtHAL3* to complement a temperature-sensitive *dfp* mutant of *E. coli*, a lethal phenotype at 42 °C (Spitzer *et al.*, 1985, 1988), was examined. The three *NtHAL3* genes were ligated into the *E. coli* expression vector pKC7 and introduced into the *dfp* mutant. Each *NtHAL3* gene could complement the *dfp* mutant strain at 42 °C (Fig. 5b). Previous studies carried out *in vitro* demonstrated that an AtHAL3 mutant protein with a His-90 to Asn change in the *P*PC-DC active site lost all *P*PC-DC activity (Kupke *et al.*, 2001). Therefore, the *P*PC-DC activity of active site mutants of NtHAL3, i.e. that NtHAL3a-H88N, NtHAL3b-H89N, and NtHAL3c-H89N are substituting the corresponding His residue by Asn, was examined by the complementation test with the *E. coli dfp* mutant. The three mutant *NtHAL3* genes did not complement the *dfp* mutation of *E. coli* (Fig. 5c). The functional difference between the yeast Hal3 and plant HAL3 was discussed (Kupke *et al.*, 2001). It was examined whether the yeast Hal3 protein had *P*PC-DC activity by a complementation test with the *E. coli dfp* mutant. As a result, yeast *HAL3* was not able to complement the *dfp* mutant of *E. coli* (Fig. 5c). These results strongly suggest that plant HAL3 proteins have *P*PC-DC activity, but yeast HAL3 protein, a known regulator of protein phosphatase activity, does not possess *P*PC-DC activity.

Overproduction of NtHAL3a *increases the intracellular ratio of proline*

Coenzyme A and its thioesters are essential cofactors for many enzymatic and energy-yielding reactions including the TCA cycle, fatty acid metabolism, and amino acid metabolism (Abiko, 1975; Tahiliani and Beinlich, 1991; Begley *et al.*, 2001). If the *P*PC-DC activity of the NtHAL3 protein functions in the metabolic pathway of coenzyme A biosynthesis from pantothenate, the intracellular concentration of some of the downstream metabolites of this pathway may possibly be increased in *NtHAL3a* overexpressing cells. Furthermore, hyperosmotic stress tolerance was improved in the transgenic BY2 cells. These results suggest the possible accumulation of amino acids that could function as a compatible solute, i.e. proline and citrulline (Delauney and Verma, 1993; Yoshiba *et al.*, 1997; Akashi *et al.*, 2001). Therefore, an attempt was made to determine the intracellullar free amino acids concentration in NtHAL3a overexpressing BY2 cells. Table 1 shows the free amino acid contents in clone SA3 of transgenic BY2 under no-stress conditions. The percentage of proline in SA3 cells increased 4.4-fold compared with that in control cells under non-stress condition (Table 1), and this increased level of proline was maintained at approximately three times higher than

Table 1. *Amino acid contents in transgenic BY2 cells*

Amino acids were extracted from transgenic BY2 cells after cultivation in modified LS medium. n=3.

Amino acid	EGFP		SA3	
	Amino acid content		Amino acid content	
	nmol g^{-1} FW	%	nmol g^{-1} FW	%
Asp	650±240	11	900±270	13
Thr	440±170	7.3	300±120	4.2
Ser	330±150	5.5	280±90	3.9
Glu	820±440	14	940±520	13
Gln	310±160	5.2	870±400	12
Gly	71±36	1.2	80±35	1.1
Ala	2100±780	35	2200±530	30
Val	820±170	14	860±110	12
Cys	ND[a]		ND	
Met	ND		ND	
Ile	81±18	1.4	130±46	1.8
Leu	170±69	2.8	190±46	2.7
Tyr	27±11	0.45	19±5	0.26
Phe	77±13	1.3	110±17	1.5
Lys	13±7	0.21	19±11	0.27
His	8.6±5	0.14	16±6	0.22
Arg	16±4	0.26	41±9	0.56
Pro	63±37	1.0	320±65	4.4
Total amino acids	6000±2300	100	7200±2200	100

[a] ND, not detected.

control even under salt-stress conditions (SA3, 4.1%; EGFP, 1.4%). The percentage of proline in another clone, SA4, was 4.9 times higher than that in control cells (data not shown).

Discussion

Three tobacco *HAL3* homologue genes, *NtHAL3a*, *NtHAL3b*, and *NtHAL3c*, were isolated with using *Arabidpsis AtHAL3* cDNA as a probe. The *NtHAL3a* gene was cloned from cultured BY2 cells, and the others were cloned from the SR-1 tobacco plant. Although BY2 and SR-1 were different cultivars, a partial cDNA fragment of *NtHAL3a* was isolated from a cDNA library of SR-1 (data not shown). Results of Southern blot analysis suggested the possibility that there are additional *HAL3* homologue genes in the tobacco plant SR-1. The expression of the *HAL3* gene was regulated by salt concentration in *Arabidopsis*, but the *NtHAL3* genes were constitutively expressed in all organs, regardless of salt-stress conditions (Fig. 3b, c) in the tobacco plant. This difference is possibly important when considering the function of *HAL3* genes in the salt tolerance of plant cells.

NtHAL3a overexpression negated the growth inhibition of BY2 cells by lithium and sodium stress (Fig. 4b, c). Lithium and sodium ion contents were examined in the transgenic cells, but these intracellular ion contents were not very different between control cells and *NtHAL3a* overexpressing cells under LiCl or NaCl stress conditions (data not shown). Therefore, overexpression of *NtHAL3a* probably does not affect ion homeostasis regulation in plant cells. It is known that sodium and lithium ion stress cause superoxide anion and free radical formation in BY2 cells (Kawano *et al.*, 2001; Hong *et al.*, 2000). *NtHAL3a* overexpressing cells showed approximately a 4–5-fold increase in the intracellular ratio of proline compared with the control cells with and without salt-stress conditions (Table 1). Previous reports proposed that proline can function as a free radical scavenger under salt and heavy metal stress conditions (Smirnoff and Cumbes, 1989; Kishor *et al.*, 1995; Nanjo *et al.*, 1999; Hong *et al.*, 2000; Siripornadulsil *et al.*, 2002). The increased level of proline caused by NtHAL3 overexpression could possibly lead to improved salt-tolerance of transgenic BY2 cells by acting as a free radical scavenger. The scavenging ability of free radicals in *NtHAL3a* expressing cells should be compared with that of control cells in future experiments.

The NtHAL3 proteins contain four conserved motifs from PPC-DC, an enzyme that functions in the coenzyme A biosynthetic pathway, and *NtHAL3* genes can complement an *E. coli* temperature-sensitive *dfp* mutation (Fig. 5b). Furthermore, active site mutant genes of *NtHAL3* could not complement the *dfp* mutation (Fig. 5c). These results proved the hypothesis, based on the results of *in vitro* experiments by Kupke *et al.* (2001), that the plant HAL3 protein functions in the coenzyme A biosynthetic pathway, by an *in vivo* experiment. Acetyl-CoA is involved in amino acid synthesis at several points including providing the carbon skeletons for leucine, a series of acetylated intermediates for the synthesis of ornithine and

arginine, or through the TCA cycle (Ireland, 1997; Thompson, 1980; Yokota *et al.*, 2002). Therefore the intracellullar free amino acid content of *NtHAL3a* transgenic BY2 cells was determined. The percentage of proline was increased about 4–5-fold in *NtHAL3a* transgenic cells with or without stress (Table.1). Proline is synthesized from both glutamate and ornithine in plant cells (Delauney and Verma, 1993), and pathway selection depends on the developmental stage of the plant and environmental stress (Delauney *et al.*, 1993; Roosens *et al.*, 1998). Acetyl-CoA is used as the first metabolic substrate in the ornithine pathway (Ireland, 1997; Thompson, 1980; Yokota *et al.*, 2002). In the *NtHAL3a* overexpressing cells, the percentage of proline was increased and that of arginine, also synthesized by the ornithine pathway, was increased approximately 2-fold (Table 1). Therefore, it is thought that the ornithine biosynthesis pathway may be strengthened by an increase in *P*PC-DC activity, and, consequently, the proline and arginine ratio was increased in *NtHAL3a* overexpressing cells. It will be important to determine whether the production level of coenzyme A and/or acetyl-CoA is increased by overexpression of *NtHAL3a* in BY2 cells. The intracellular localization of NtHAL3 proteins will be important information for understanding the proper use of each NtHAL3 in tobacco cells. Based on the present study, it appears that plant *HAL3* genes are not only important for the molecular breeding of salt-tolerant plants, but also for the metabolic regulation of coenzyme A biosynthesis from pantothenate and its effects on the biosynthesis of several amino acids through the ornithine pathway in plant cells.

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